CR 92.008

NCEL

Contract Report

June 1992

An Investigation Conducted by
Dr. Eve Riser-Roberts

IN SITU/ON-SITE BIODEGRADATION OF REFINED OILS AND FUELS (A Technology Review)

Volume 1

Abstract This extensive literature review covers all aspects of in situ and on-site bioremediation of gasoline, middle distillate fuels, and other refined petroleum products, using information available through 1988. Topics covered include: composition of different fuels, factors affecting biodegradation rates, responsible microorganisms, degradation pathways, factors enhancing biodegradation, and potential limitations. This report is in three volumes. Volume 1 covers general information of most interest to managers and non-specialists. Volume 2 contains technical information required for in-depth coverage. Volume 3 covers related information, including detailed applied information on screening and microbial enumberation methods, laboratory microorganisms studies, bioreclamation site preparation, bioreactor use and selection, cost data, and competing technologies.

NAVAL CIVIL ENGINEERING LABORATORY PORT HUENEME CALIFORNIA 93043-5003

METRIC CONVERSION FACTORS

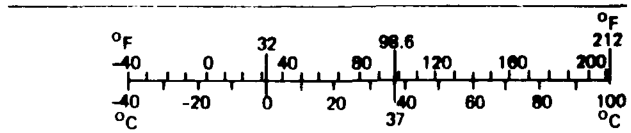
Approximate Conversions to Metric Measures

Symbol	When You Know	Multiply by	To Find	Symbol
		LENGTH		
in	inches	*2.5	centimeters	cm
ft	feet	30	centimeters	cm
yd	yards	0.9	meters	m
mi	miles	1.6	kilometers	km
		AREA		
in^2	square inches	6.5	square centimeters	cm^2
ft^2	square feet	0.09	square meters	m^2
yd^2	square yards	0.8	square meters	m^2
mi^2	square miles	2.6	square kilometers	km^2
	acres	0.4	hectares	ha
		MASS (weight)		
oz	ounces	28	grams	g
lb	pounds	0.45	kilograms	kg
	short tons (2,000 lb)	0.9	tonnes	t
		VOLUME		
tsp	teaspoons	5	milliliters	ml
Tbsp	tablespoons	15	milliliters	ml
fl oz	fluid ounces	30	milliliters	ml
c	cups	0.24	liters	l
pt	pints	0.47	liters	l
qt	quarts	0.95	liters	l
gal	gallons	3.8	liters	l
ft^3	cubic feet	0.03	cubic meters	m^3
yd^3	cubic yards	0.76	cubic meters	m^3
		TEMPERATURE (exact)		
°F	Fahrenheit temperature	5/9 (after subtracting 32)	Celsius temperature	°C

*1 in = 2.54 (exactly). For other exact conversions and more detailed tables, see NBS Misc. Publ. 286, Units of Weights and Measures, Price $2.25, SD Catalog No. C13.10.286.

Approximate Conversions from Metric Measures

Symbol	When You Know	Multiply by	To Find	Symbol
		LENGTH		
mm	millimeters	0.04	inches	in
cm	centimeters	0.4	inches	in
m	meters	3.3	feet	ft
m	meters	1.1	yards	yd
km	kilometers	0.6	miles	mi
		AREA		
cm^2	square centimeters	0.16	square inches	in^2
m^2	square meters	1.2	square yards	yd^2
km^2	square kilometers	0.4	square miles	mi^2
ha	hectares (10,000 m^2)	2.5	acres	
		MASS (weight)		
g	grams	0.035	ounces	oz
kg	kilograms	2.2	pounds	lb
t	tonnes (1,000 kg)	1.1	short tons	
		VOLUME		
ml	milliliters	0.03	fluid ounces	fl oz
l	liters	2.1	pints	pt
l	liters	1.06	quarts	qt
l	liters	0.26	gallons	gal
m^3	cubic meters	35	cubic feet	ft^3
m^3	cubic meters	1.3	cubic yards	yd^3
		TEMPERATURE (exact)		
°C	Celsius temperature	9/5 (then add 32)	Fahrenheit temperature	°F



REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*

2. REPORT DATE
June 1992

3. REPORT TYPE AND DATES COVERED
Final; January 1987 - November 1988

4. TITLE AND SUBTITLE
IN SITU/ON-SITE BIODEGRADATION OF REFINED OILS AND FUELS (A Technology Review)

5. FUNDING NUMBERS
PR - 71-033
C - N68305-6317-7115
WU - DN668034

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER
CR 92.008

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Naval Facilities Engineering Command
Cheaspeake Division
Washington Navy Yard
Washington, DC 20374-2121

/ Naval Civil Engineering Laboratory
Code L71
Port Hueneme, CA 93043

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This extensive literature review covers all aspects of in situ and on-site bioremediation of gasoline, middle distillate fuels, and other refined petroleum products, using information available through 1988. Topics covered include: composition of different fuels, factors affecting biodegradation rates, responsible microorganisms, degradation pathways, factors enhancing biodegradation, and potential limitations. This report is in three volumes. Volume 1 covers general information of most interest to managers and non-specialists. Volume 2 contains technical information required for in-depth coverage. Volume 3 covers related information, including detailed applied information on screening and microbial enumberation methods, laboratory microorganisms studies, bioreclamation site preparation, bioreactor use and selection, cost data, and competing technologies.

14. SUBJECT TERMS
Bioremediation, in situ, fuels, on-site, biodegradation, literature review

15. NUMBER OF PAGES
562 (all three volumes)

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. 239-18
298-102

ACKNOWLEDGMENTS

This technology review was conducted under the auspices of the Naval Civil Engineering Laboratory (NCEL) on Purchase Order Number N68305-6317-7115, in connection with the installation restoration effort at Patuxent River Naval Air Station (NAS Patuxent River), MD. The author wishes to acknowledge the helpful input of Mr. R. Hoeppel, NCEL Project Engineer, and Dr. M. Swindoll (previously of NCEL), as well as that of Dr. D.L. Sorensen and Dr. R.R. Dupont, of the Utah Water Research Laboratory, who reviewed the manuscript.

Information for this report was obtained, in part, through API, NTIS, DTIC, and Dialogue searches, and by extensive use of the library facilities at the University of California at Santa Barbara.

PREFACE

This technology review has been prepared in response to the spillage and chronic leakage of fuel oil from underground tanks on the fuel farm at the Patuxent River Naval Air Station (NAS Patuxent River), MD. This review will provide background information on the major aspects of technologies and related research dealing with the use of biodegradation for treatment of environmental contamination by toxic organic substances. It is intended to serve as a broad reference base for development of a program for *in situ* biorestoration of the fuel-contaminated soil and groundwater at the Patuxent River Naval Air Station and at other similarly contaminated Navy sites.

This report was written in 1988, but because of financial constraints, the review process was not completed until 1991. There has been no update of data resulting from work performed in the intervening years. It is hoped that this document will, nevertheless, serve as a valuable source of background information on the use of biodegradation as a promising treatment alternative for incidents involving environmental contamination by petroleum products.

The report is organized into three volumes:

Volume 1 contains the main text, which is an overview of the many different subject areas involved in bioremediation. This summary is intended for managers and others who wish to learn about the technology without having to confront the technical details.

Volume 2 is Appendix A, Supplementary Text, which contains the technical information for readers who need indepth coverage. The section headings in Volume 1 correspond to those in Volume 2 to allow convenient referral to the supplementary text when more details are required.

Volume 3 contains Appendices B through F, which address related subject matter.

DISCLAIMER

Reference herein to any company, commercial product, equipment, procedure, program, process, or service does not constitute or imply its endorsement, recommendation, or approval by the author or the Naval Civil Engineering Laboratory.

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GLOSSARY

Term/Acronym	Description
Aerobic	In the presence of oxygen
Anaerobic	In the absence of oxygen
Anthropogenic	Of man-made origin
Autochthonous	Indigerous or native bacteria found in soil in relatively constant numbers that do not change rapidly in response to the addition of specific nutrients
Autotrophic	The ability to fix carbon for growth from carbon dioxide and obtain energy from light or oxidation of inorganic compounds
Bioaugmentation	See Enhanced biodegradation
Biodegradation	Breakdown of organic substances by microorganisms
Bioreclamation	Use of biodegradation to restore soil and groundwater to uncontaminated condition
BDAT	Best demonstrated available technology
BOD	Biochemical oxygen demand
BTX	Benzene, toluene, and xylenes
CEQ	Council on Environmental Quality
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act (Superfund)
CGA	Colloidal gas aprons
COD	Chemical oxygen demand
Commensalism	Sequential degradation of a compound by two or more microorganisms in a relationship that may benefit only one partner
Cometabolism or Cooxidation	The indirect metabolism of a recalcitrant substance; the process by which microorganisms, in the obligate presence of a growth substrate, transform a nongrowth substrate
Cyanobacteria	Blue-green algae, which are actually bacteria
Denitrifying bacteria	Bacteria that reduce nitrate using the oxygen of nitrate as a hydrogen acceptor; requires source of hydrogen and limitation on supply of free oxygen
DOC	Dissolved organic carbon
DOD	Department of Defense
E^0	Standard reduction potential
EPA	Environmental Protection Agency
Enhanced biodegradation	Stimulation of microbial degradation of organic contaminants by addition of microorganisms, nutrients, or optimization of environmental factors on-site or <u>in situ</u>
Enrichment culturing	Addition of a specific hydrocarbon to a minimal medium to select for degraders of that compound
Eukaryotic	Nucleus is surrounded by a membrane, as in fungi and higher organisms
Facultative	The ability to adapt to the conditions specified with this term
Facultative anaerobes	Microorganisms that are metabolically active under aerobic/anaerobic conditions
Heterotrophic	The ability to derive energy and carbon for survival and growth from decomposition of organic materials
HSWA	Hazardous and Solid Waste Amendments

GLOSSARY (continued)

Term/Acronym	Description
Hydrocarbonoclastic	Ability to degrade and utilize hydrocarbons
In situ bioreclamation	Biodegradation operations taking place in the contaminated soil or groundwater without excavation or withdrawal
In vitro	In a test tube
In vivo	In life
Lithotrophic	The ability to obtain energy from oxidation of inorganic compounds
Mesophilic	The ability to grow at temperatures from 10° to $45^\circ C$, with optimum growth around 20° to $40^\circ C$; most human pathogens grow best at $37^\circ C$
Methanogenic consortia	Groups of microorganisms that function under highly reducing conditions and produce methane from degradation of small or low molecular weight organic compounds
Microaerophilic	The ability to survive on very low levels of oxygen
Mineralization	Complete biodegradation of organic molecules to mineral products
MLSS	Mixed liquor suspended solids
Nitrogen demand	Amount of nitrogen required for degradation of a given amount of contaminant
NRV	Nitrogen requirement value; the amount of nitrogen required by organisms to decompose or degrade a particular organic chemical
Normal flora	Mixed population of microorganisms occurring in nature
NRV	Nitrogen requirement value
Obligate	Strict dependence upon the conditions specified with this term
Oligotrophic	The ability to survive on very low concentrations of nutrients
On-site bioreclamation	Biodegradation operations that occur above ground at the site of contamination
Operon	A DNA region that codes for several enzymes in a reaction pathway; it enables or prevents repression of structural gene function by controlling synthesis of mRNA by RNA polymerase enzyme
OTA	Congressional Office of Technology
PAHs	Polycyclic aromatic hydrocarbons, also called polyaromatic hydrocarbons and PNAs
PCBs	Polychlorinated biphenyls
Photosynthetic	The ability to obtain energy from light
Pleomorphs	Bacteria having multiple shapes
PNAs	Polynuclear aromatic hydrocarbons, also called PAHs
Procaryotic	"Nucleus" is a single chromosome without a membrane, as in bacteria
Psychrophilic	The ability to grow best at temperatures from -5° to $30^\circ C$, with optimum growth between 10° and $20^\circ C$
RAS	Return activated sludge
RBC	Rotating biological contactor
RCRA	Resource Conservation and Recovery Act
Recalcitrant	Resistant to microbial degradation

GLOSSARY (continued)

Term/Acronym	Description
SARA	Superfund Amendments and Reauthorization Act
SITE	Superfund Innovative Technology Evaluation Program
Sulfate-reducing bacteria	Bacteria that use sulfate as a hydrogen acceptor, converting sulfate to sulfide
Superfund	See CERCLA
Tetren	Tetraethylenepentamine, a chelator
TSDFs	Treatment Storage and Disposal Facilities
Thermophilic	The ability to grow at temperatures from 25^{o} to 80^{o}C, with optimum growth at 50^{o} to 60^{o}C
Turnover time	The amount of time required to remove the concentration of substrate present
Vadose zone	Unsaturated soil above water table
Xenobiotic	Compounds that are man-made or are unique in nature
Zymogenous	Soil bacteria that increase rapidly when furnished with certain nutrients and then diminish in numbers when the material is exhausted

SECTION 1

INTRODUCTION

1.1 BACKGROUND

1.1.1 Environmental Contamination

Environmental pollutants are defined as chemicals of natural or synthetic origin that are released by man's activity into the environment where they have an undesirable effect on the environment or on man *via* the environment (Hutzinger and Veerkamp, 1981). There is a growing concern in the United States as a wide variety of synthetic organic chemicals are being introduced inadvertently or deliberately into waters and soils (Alexander, 1980). More than five million chemical compounds were described in Chemical Abstracts by the year 1980 (Ghisalba, 1983). This does not necessarily encompass all naturally occurring substances, nor does it include those resulting from the degradation of synthetic chemicals released to the environment (Richards and Shieh, 1986). Some 70,000 substances are in U.S. commerce (Ghisalba, 1983). Around 1000 new chemicals are brought on the market annually, while 150 chemicals are produced in excess of 50,000 tons per year. The total world production of synthetic organic chemicals is estimated at 300 million tons/yr. As a by-product of industrial production, some 265 metric tons of hazardous waste are generated every year by 14,000 U.S. industrial plants (Nicholas, 1987). The U.S. Environmental Protection Agency (EPA) and the Congressional Office of Technology Assessment (OTA) estimate that 80 percent of this waste is disposed of in landfills. Many of these wastes are toxic and persistent in the environment. Ecotoxicological data are available for less than 1000 compounds (Ghisalba, 1983). The dangers arising from the environmental exposure to such chemicals and their decomposition are not known for most of them (Schmidt-Bleek and Wagenknecht, 1979).

A nationwide survey conducted for the EPA indicated that organic compounds are the most common environmental contaminants, being found at 358 of the 395 hazardous waste sites included in the survey (Steelman and Ecker, 1984). Another study was made of the contaminants at 114 high priority Superfund sites (Ellis, Payne, and McNabb, 1985). The classes of chemical wastes found at the greatest number of sites, in order of decreasing prevalence, were: slightly water soluble organics (e.g., aromatic and halogenated hydrocarbon solvents, chlorophenols), heavy metal compounds, and hydrophobic organics (e.g., polychlorinated biphenyls, aliphatic hydrocarbons).

Organic solvents were reported at nearly 28 percent of all hazardous waste sites (JRB and Associates, Inc., 1984b). These solvents appear to degrade only slowly, if at all, once they enter the groundwater. Hydrocarbon compounds, such as fuels, lubricating oils, and creosote, were found at nearly 15 percent of hazardous waste sites. Some of these contaminants are toxic (Alexander, 1980). Naphthalene and its methyl-substituted derivatives are some of the most acutely toxic, water-soluble components of crude oils (Anderson, Neff, Cox, Tatem, and Hightower, 1974). As the molecular size of polyaromatic hydrocarbons (PAHs) increases up to four or five fused benzene rings, their lipophilicity, environmental persistence, and mutagenicity also increase

(Jacob, Karcher, Belliardo, and Wagstaffe, 1986; Miller and Miller, 1981). Some PAH's may be converted to toxic substances in the environment (Alexander, 1980). Many represent classes of molecules not previously investigated, and some have no close structural analogs in nature. Many have been intentionally developed to be resistant to microbial attack, and, not surprisingly, tend to persist in nature.

Chemicals may enter the environment directly as a result of accidents, spills during transportation, leakage from waste disposal or storage sites, or industrial facilities (Ghisalba, 1983). Oils and fuels generally enter the environment as a result of leaking storage tanks and associated piping (both above and below ground) or accidental spills (JRB and Associates, Inc. 1984b). A sudden and increasing frequency of tank failures has been experienced from the leaking of gasoline from underground gasoline storage tanks, primarily at automobile service stations. Steel tanks have an average life expectancy of 25 years and, after their first 20 years, have demonstrated an increase in the rate of test failure with age (Robison, 1987). It is estimated that of the approximately 1.4 million buried gasoline and fuel tanks, 75,000 to 100,000 are currently leaking, and the final count could be as high as 350,000 (Brown, Norris, and Brubaker, 1985). The EPA estimates that service station gasoline tanks alone could be losing 11 million gal/yr of product.

Actual tank leaks are only a small part of the problem. Freezing and rain affect the surrounding soils and create stresses that cause buoyancy and the shifting of pipes and other installations. Because of these stresses a fueling system can actually start leaking from the day it is installed. In pipeline accidents, 157,760 barrels of gasoline and crude oil were lost to the environment in 233 reported incidents in 1971 (Meyer, 1973). Added to this are the unreported or undetected leaks, e.g., well casings and tanks.

For decades, the Department of Energy (DOE) and its predecessor agencies have engaged in a wide range of operations that generate organic chemical wastes (Steelman and Ecker, 1984). The vast majority of Department of Defense (DOD) hazardous waste sites are contaminated with organic chemicals, the most common being fuels and chlorinated solvents. Past disposal practices have led to migration of organics from the disposal sites <u>via</u> the groundwater pathway. DOD sites are most often contaminated by underground tank and line leaks, sludge burial pits, chemical evaporation pits, fire training areas, and landfills. The DOD is developing <u>in situ</u> treatment technologies as part of a general effort to determine the most effective and least costly methods to clean up its hazardous waste sites.

Subsurface contamination is complex and difficult to treat (FMC Aquifer Remediation Systems, 1986). The soil is particularly difficult to treat because of the tendency for contaminants to tightly bind or sorb to the soil particles. Soil contamination is also generally more severe than groundwater contamination, ranging from a few thousand ppm to greater than 10 to 15 percent by weight in the soil, as compared with a maximum of a few hundred ppm in groundwater.

It is very difficult to estimate the quantity and scope of hydrocarbon contamination from spills that have been reported (Raymond, Jamison, and Hudson, 1976). In spills involving light and more soluble hydrocarbons, e.g., gasoline, the adsorbed product could slowly leach into a water supply for many

years, if the removal processes were not enhanced. Ultimately, the result of subsurface soil contamination is groundwater contamination (FMC Aquifer Remediation Systems, 1986)

1.1.2 Groundwater Contamination

Groundwater is water present in the soil within a zone of saturation; it is generally derived from precipitation or stream infiltration (Bitton and Gerba, 1985). Water-bearing formations that will transmit and yield significant quantities of water to wells and springs are called aquifers. Within the zone of saturation, groundwater occurs under either water table or artesian conditions. Under water table conditions, the groundwater is not confined. The upper surface of the saturated zone is called the water table and may be near the land surface or hundreds of feet below it. The water table rises and falls with changes in volume. Under artesian conditions, the groundwater is confined between an upper and lower confining bed. Water in a well will then rise above the bottom of the upper confining bed. Some background information about groundwater is given in Table 1.

In recent years, there have been many cases of groundwater contamination (Wilson, McNabb, Balkwill, and Ghoirse, 1983). Contamination of groundwater with hydrocarbons is a very common problem in the United States (Brown, Loper, and McGarvey, 1986). A study by the Council on Environmental Quality (CEQ) showed that major groundwater contamination problems in many states were caused by synthetic organic chemicals resulting from industrial and manufacturing operations (Ellis, Payne, and McNabb, 1985). Contamination of groundwater by these compounds was a major problem in at least 34, and possibly as many as 40, states (Steelman and Ecker, 1984). The problems exist in all states east of the Mississippi River and in many less industrialized states, such as Arizona and Idaho. Once an aquifer used as a water supply is contaminated, 100 percent of its available groundwater must be considered contaminated. Environmental "watchdog" organizations are demanding that if a groundwater supply is contaminated, it must be restored (Brown, Loper, and McGarvey, 1986).

Groundwater is an important natural resource (Brown, Loper, and McGarvey, 1986), constituting approximately two-thirds of the world's freshwater. Its use has increased 250 percent from 1950 to 1980, with over 50 percent of the population depending upon it as a source of drinking water. A portion of the U.S. population drinks untreated groundwater. In Florida, for instance, almost 92 percent of the population depends upon groundwater for drinking, and 20 percent of those receive untreated groundwater (Florida Environ. Service Center, 1982).

Groundwater is also a vulnerable resource. Its protection becomes critical as more and more of the surface waters are contaminated and greater reliance is placed on groundwater for domestic, agricultural, and industrial use (Florida Environ. Service Center, 1982). It is estimated that about 1 percent of the nation's groundwater supply is currently contaminated to some degree (Josephson, 1983a). One-third of all large public water systems have man-made contamination and over 7,000 private, public, and industrial wells have been closed or seriously affected by contamination from 1980 to 1986.

Groundwater is a fragile resource; once contaminated, it is difficult to remediate (Steelman and Ecker, 1984). Contaminants can remain in concentrated

Table 1. Facts About Groundwater (Bitton and Gerba, 1985)

Some Parameters:	
Age	Few hours to thousands of years
Temperature	Increases with depth (c.$3^{o}C$/100 m)
Conductivity	Presence of dissolved ions (e.g., Na^+, $Ca^=$, Mg^{2+}, Cl^-, SO_4^{2-}, NO_3^-...) Concentration: hundreds of mg/l
Hydrostatic pressure	Increases with depth (1 atm/10 m)
Major Sources of Groundwater Contamination (USA)	Domestic Water 3 billion M^3/yr More than 0.6 million wells receive 850 billion gal/yr Industrial Sources 100,000 abandoned industrial waste sites 75,000 active industrial landfills 173,000 waste lagoons Agricultural Practices Pesticides and fertilizers
Groundwater as a Resource (USA)	25% of all water used
Groundwater Uses (USA)	Irrigation 69% Industry 14% Urban and rural drinking 17%
Groundwater for Drinking (USA)	Supplies the needs of 50% of US population. Supplies 95% of drinking needs in rural areas. U.S. public water systems: 75% rely on ground-water
Major Public Health Concerns	Waterborne Diseases: 54% of diseases occurred in noncommunity water systems relying solely on groundwater. Inorganic and Organic Chemicals: Concern about toxic, mutagenic, carcinogenic, and teratogenic compounds. Not well documented.
Groundwater Legislation	Groundwater quality addressed in numerous pieces of legislation. Acts must be coordinated.

areas of the groundwater for long periods of time (Ahlert and Kosson, 1983). The average residence time of a pollutant in groundwater is 200 years (Meyer, 1973). Once aquifers are contaminated, it can be very difficult and costly to restore them to their original quality. The treatment will probably be time consuming and expensive, with costs ranging from tens of thousands of dollars for simple treatment programs, up to tens of millions of dollars for complex, large sites (Lee and Ward, 1986). Groundwater also supplies 40 percent of the nation's irrigation water (Plumb, 1985). Yet, despite the importance of groundwater and the potential effect that contamination could have on its use, there has been no concerted effort to define the impact of the estimated 20,000 to 50,000 waste disposal sites in existence on this resource.

Groundwater may contain a wide range of organic substances, some of which are of natural (e.g., humic and fulvic acids) and some of anthropogenic (man-made) origin (Bitton and Gerba, 1985). Many chemicals now found in groundwater have not yet been tested for health effects, and no standards exist for their maximum concentration in water (Pye and Patrick, 1983). Some man-made chemicals are resistant to microbial degradation in the soil and reach the groundwater (Bitton and Gerba, 1985). The occurence of these compounds in groundwater supplies has raised concern over their toxicity, mutagenicity, teratogenicity, and carcinogenicity.

Groundwater contamination is a complex phenomenon (FMC Aquifer Remediation Systems, 1986). Material, such as gasoline, will enter the subsurface, travel through the vadose zone (the unsaturated soil), and leave behind soil containing residual contamination. When the material reaches the water table or an impermeable medium (bedrock, clay), it spreads out on the surface (Texas Reserch Institute, Inc., 1982). If the material is less dense than water, such as petroleum hydrocarbons, it will form a "pool" of mobile free-phase liquid that travels along the groundwater table following the hydraulic gradient (FMC Aquifer Remediation Systems, 1986). The water-soluble components of the gasoline begin to dissolve in the water, contaminating a water resource (Texas Research Institute, Inc., 1982). As the water table rises and falls, the free phase material will be smeared within the soil, resulting in a wide band of adsorbed hydrocarbon (FMC Aquifer Remediation Systems, 1986). Water in contact with the free phase and adsorbed contaminant will leach hydrocarbons creating a soluble plume of contamination that travels downgradient. Rain water continues to contribute to the leaching of waste components into the aquifer, as it percolates through the gasoline-contaminated area. This will be a problem as long as any residual product remains in the soil (Texas Research Institute, Inc., 1982).

In gasoline spills, the monoaromatic hydrocarbons have relatively high pollution potential based upon their concentration in gasoline, their aqueous solubility, and their estimated toxicity or carcinogenicity (Barker and Patrick, 1985). The monoaromatics of particular concern are the water-soluble and mobile gasoline components, referred to as BTEX; i.e., benzene, toluene, ethylbenzene, and the xylene isomers, m-, p-, and o-xylene. It is likely that gasoline spills will have the most serious and immediate impact upon highly permeable, shallow, water table aquifers. Such aquifers are also favored for private and municipal drinking water supplies.

The impacts of losing hydrocarbons to the subsurface will vary (FMC Aquifer Remediation Systems, 1986). Fumes from free product and contaminated soils

will migrate up through the vadose zone and can collect in basements, creating explosion hazards. Leaching of hydrocarbons will impact the quality and use of the groundwater. With extreme losses of material, the free product layer can actually seep to the surface in groundwater discharge areas.

Most site investigations have been confined to the property, or near vicinity of the suspected source of contamination. However, an extensive analysis of water supply wells by the Council on Environmental Quality (CEQ) in 1981 (Council on Environmental Quality, 1981) indicated that the migration of contaminant plumes is more widespread than this. An example involved a contaminant plume in Nashua, NH, that was 1500 ft long, 110 ft deep, and covered an area of about 1.29×10^6 sq ft (Josephson, 1983b). The estimated movement of the plume was about 1.7 ft/day.

Groundwater in the vicinity of hazardous waste disposal facilities may become contaminated with a larger number of chemicals than previously thought (Plumb, 1985). Monitoring data from 358 hazardous waste disposal sites identified 720 substances, including 558 organic compounds, that have been reported in the groundwater at one or more sites. The organic compounds established by the EPA as priority pollutants are listed in Table A.1-1. Most classes of priority pollutants (volatiles, base/neutrals, acid extractables, pesticides, and inorganics) have been detected in the groundwater in the vicinity of disposal sites. However, since this information has never been centralized, contamination from hazardous waste disposal sites has been treated as a series of isolated examples rather than symptoms of a more extensive problem. By comparison, 1,259 contaminants have been reported in surface water (Shackelford and Keith, 1977). The organic priority pollutants have been detected in the groundwater of all 10 EPA regions (Plumb, 1985). The most commonly encountered are listed in Table A.1-2. Volatile compounds have been identified most frequently and at the greatest concentrations.

There are more than 200,000 various types of surface impoundments that are potential future sources of pollution (FMC Aquifer Remediation Systems, 1986). In 1978, the EPA estimated there were 25,000 active industrial impoundments and landfills in the USA and that many of these could impact usable aquifers (Pye, Patrick, and Quarles, 1983). Many of these sites may require remedial action to restore the groundwater to usable condition. An estimated 50 to 60 percent of the landfills with interim status under the Resource Conservation and Recovery Act (RCRA) may be contaminating groundwater, including several RCRA facilities that have received materials from Superfund cleanups (Hileman, 1984).

Groundwater has been assumed to be pristine due to the protective role of the overlying soil mantle (Thornton-Manning, Jones, and Federle, 1987). In reality, it is as contaminated as surface waters in some areas (Page, 1981).

Unlike streams and rivers, groundwater moves very slowly (Brown, Loper, and McGarvey, 1986). Its rate and direction of flow are influenced by factors, such as substrate composition, recharge rates, and gravity. It has been widely held that once groundwater is contaminated, it is permanently lost. However, groundwater systems do, in fact, have mechanisms to regenerate themselves. Contaminant transport is a dynamic process. Natural biodegradation is an active phenomenon; however, biodegradation mechanisms are limited by high levels of contamination. Nevertheless, biodegradation offers an alternative

strategy for dealing with groundwater contamination by accelerating attenuation through in situ treatment.

1.1.3 Pollution Legislation

The June 1976 Consent Decree of the National Resources Defense Council resulted in the U.S. Environmental Protection Agency (EPA) identifying 65 classes of toxic chemicals (Richards and Shieh, 1986). This toxic pollutants list presented the core pollutants for which pretreatment standards had to be established under the Clean Water Act of 1977. The list comprised thousands of individual chemicals and proved too voluminous as a guideline for chemical analyses to be performed on environmental samples and industrial effluents (Leisinger, 1983). Therefore, the list was reduced to 129 compounds and elements (114 organic compounds plus cyanide, asbestos, and 13 metals) from the 65 pollutant classes (Keith and Telliard, 1979). These compounds and elements are referred to as "priority pollutants." In 1979, the U.S. EPA published the original two-volume report Water-Related Environmental Fate of 129 Priority Pollutants (1979) (Environmental Protection Agency, 1979), which identified the nine pollutant groups of concern. These included aliphatics, halogenated aliphatics, nitrosamines, aromatics, chloroaromatics, polychlorinated biphenyls (PCBs), nitroaromatics, polynuclear aromatic hydrocarbons, and pesticides (see Table A.1-1).

Proper management of hazardous waste has become a top priority of Government officials, industrial managers, and private citizens (Scholze, Wu, Smith, Bandy, and Basilico, 1986). Since 1976, considerable effort has been expended toward this goal by identifying the origin of hazardous wastes, developing appropriate technologies for management of these wastes, and implementing regulatory schemes that provide greater protection of public health and the environment. However, for many large users, the threat of liability is more powerful than any regulations that can be written (Robison, 1987).

There are numerous institutional limitations that can affect if, when, what, and how remediation will be selected and carried out (Wilson, Leach, Henson, and Jones, 1986). Groundwater remediation may require compliance with a multitude of local, state, and Federal pollution control laws and regulations. If the response involves handling hazardous wastes, discharging substances into the air or surface waters, or the underground injection of wastes, Federal pollution laws apply. These laws do not exempt the activities of Federal, state, or local officials or other parties attempting to remediate contamination events. They apply to generators and responding parties alike, and it is not unusual for these pollution control laws to conflict. For example, a hazardous waste remediation project may be slowed, altered, or abandoned by the imposition, upon the party undertaking the effort, of elaborate RCRA permit requirements governing the transport and disposal of hazardous wastes.

Federal Regulations

Environmental laws pertinent to in situ bioreclamation of fuel oil are the Resource Conservation and Recovery Act (RCRA), Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), Superfund Amendments and Reauthorization Act (SARA), the Clean Water Act, and the Safe Drinking Water Act (Public Law 99-499, 1986). RCRA was passed by Congress in 1976 to begin to

cope with unsafe disposal of hazardous waste (Nicholas, 1987). RCRA mandated the establishment of a "cradle to grave" program for safe disposal of hazardous waste and directed EPA to adopt criteria for identifying hazardous waste and to establish standards for handling and disposal of hazardous waste by generators, transporters, and disposers. Operators of hazardous waste generators and Treatment Storage and Disposal Facilities (TSDFs) were required to obtain permits from EPA. Congress attempted to force alternative solutions to landfills by making the use of landfills expensive. RCRA also provided more direct incentives for resource conservation and recovery to reduce the volume of waste and to facilitate development of alternative technologies.

Under the Safe Drinking Water Act of 1974, EPA was directed to establish national drinking water standards to protect public health (Miller and Miller, 1981). This act contains an underground injection control program to prevent groundwater contamination by waste injection wells and to protect sole-source aquifers for drinking water (Pye and Patrick, 1983).

The Clean Water Act of 1977 requires the EPA to establish, equip, and maintain a water quality surveillance system for groundwater, as well as surface water (Pye and Patrick, 1983). The Toxic Substances Control Act of 1976 and the Surface Mining Control and Reclamation Act both have provisions that could offer a measure of protection for groundwater.

The Hazardous and Solid Waste Amendments (HSWA) were passed to reduce the dependence upon land disposal of hazardous wastes (Scholze, Wu, Smith, Bandy, and Basilico, 1986). As a result, the EPA determined which wastes should be restricted from land disposal unless treated. The EPA is considering a proposal to establish performance standards for treatment of all listed hazardous wastes and would have to identify the Best Demonstrated Available Technology (BDAT) for a particular listed waste category. The highest achievable performance level for that BDAT process would become the standard for that waste. Waivers for specific wastes would require use of the next BDAT for that waste. Two BDAT performance standards may have to be established: one for concentrated and one for dilute wastes. The former would continue to be incineration for most organic constituents and some type of solidification for inorganics. The latter may be based on biological treatment, which would provide many opportunities for commercial development of a wide range of microbial applications.

In 1980, Congress passed CERCLA, commonly referred to as Superfund. CERCLA authorizes the Federal Government to clean up contamination caused by inactive waste disposal sites or spills, many of which pose immediate threats to groundwater quality (Pye and Patrick, 1983). Superfund put the burden on the generator, transporter, and disposer to clean up existing, nonpermitted disposal sites. It established a mechanism to identify sites that present significant threats to health or to the environment; it required EPA to develop a list of high-priority sites for cleanup; it empowered EPA to clean up the priority sites, if the responsible party was unidentifiable; it created an emergency response fund, with a small Federal contribution and a tax on petrochemical feedstocks and certain other chemicals, for EPA cleanups; and it authorized EPA to recover cleanup costs from the responsible parties. The original Superfund program was established for five years at a cost of $1.1 billion. RCRA and Superfund were to provide a strong national mandate for the development of alternative ways to eliminate or lessen the toxicity and volume of hazardous waste.

In 1980, EPA proposed a national groundwater strategy (Environmental Protection Agency, 1980). Its goal was to prevent groundwater contamination rather than provide remedial action. The suggested approach included the development of state management and protection strategies; the development of a groundwater classification system; and EPA coordination of existing Federal programs for groundwater protection. The proposed strategy was aimed at protecting groundwater quality according to its value and use, and the technical approach adopted included the use of siting and design criteria, best management practices, effluent standards, innovative and alternative technologies to achieve performance standards, and, to a lesser extent, numerical groundwater quality standards and economic incentives.

In 1981, because of its lack of action on the proposed national groundwater strategy, EPA was compelled to create two separate groundwater task forces, one for policy and one for technical purposes, to develop a consistent agencywide strategy. This objective has still not been realized and the EPA is revising its groundwater policy statement.

The EPA has also established a formal program to enhance the development and use of new or innovative technologies for mitigating the problems caused by releases of hazardous substances at uncontrolled hazardous waste sites (Sanning and Olfenbuttel, 1987). In the U.S., the program is called the Superfund Innovative Technology Evaluation, or SITE, program. In 1986, the NATO-CCMS formally adopted a U.S. proposal for a new pilot study entitled, "Demonstration of Remedial Action Technologies for Contaminated Land and Groundwater." The following NATO countries are participating: Canada, Denmark, Federal Republic of Germany, Greece, Italy, The Netherlands, Norway, Spain, and the United States. Two non-NATO countries, Australia and Japan, have also expressed an interest in participating. This study is a logical international extension of the U.S. EPA SITE program and will field demonstrate and evaluate new technology or existing systems for remedial action at uncontrolled hazardous waste sites.

SARA is the Superfund Amendments and Reauthorization Act of 1986, which extends and amends CERCLA. On a Federal level, not later than 18 months after the enactment of SARA, the Administrator must take steps to assure that a preliminary assessment is conducted for each facility on the Federal Agency Waste Compliance Docket (Public Law 99-499, 1986). The facilities must be evaluated in accordance with the criteria established, in accordance with Section 104 under the National Contingency Plan for determining priorities among releases, and these facilities must be included on the National Priorities List.

On a state level, not later than six months after the inclusion of any facility on this list, the department, agency, or instrumentality responsible for such facility must begin a remedial investigation and feasibility study for the facility (Public Law 99-499, 1986). Within 180 days after the results of an investigation and study are reviewed, an agreement must be reached to complete all remedial action necessary. Substantial continuous physical on-site remedial action must begin not later than 15 months after completion of the investigation and study.

The 1984 amendments to RCRA and the 1986 reauthorization of Superfund reaffirm the policy of limiting land disposal of hazardous waste and of sup-

porting the development of alternative technologies (Nicholas, 1987). According to the 1984 RCRA amendments, "certain classes of land disposal facilities are not capable of assuring long-term containment of certain hazardous wastes, and to avoid substantial risk to human health and the environment, reliance on land disposal should be minimized or eliminated, and land disposal, particularly landfill and surface impoundment, should be the least favored method for managing hazardous wastes." The act declares as a national policy "minimizing the generation of hazardous waste and the land disposal of hazardous waste."

Amendments to Superfund call for establishment of major new programs to stimulate waste reduction and development and demonstration of new technologies (Nicholas, 1987). The amendments authorize three new programs at a cost of approximately $250 million over the next five years. They establish a fund, administered by the National Institute of Environmental Health Sciences, for basic research and training grants. They also create a new office at EPA, called the Office of Technology Demonstration, to direct the "research, evaluation, testing, development, and demonstration of alternative or innovative technologies to be used at Superfund sites." And they call for the establishment of at least five university-based centers to conduct research and training on various aspects of hazardous waste disposal.

In situ remediation procedures may be subject to permitting or other requirements of Federal or state underground injection control programs (Wilson, Leach, Henson, and Jones, 1986). Withdrawal and treatment approaches may be subject to regulation under Federal or state air pollution control programs or to pretreatment requirements, if contaminated groundwater will be discharged to a municipal wastewater treatment system. Pumping from an aquifer may involve a state's groundwater regulations on well construction standards and well spacing requirements, as well as interfere with various competing legal rights to pump groundwater. Other factors influencing remediation decisions are the availability of alternate sources of water supply, political and legal pressures, and the availability of funds.

By executive order, all Federal agencies must comply with the letter and intent of all Federal, state, and local environmental regulations (Heyse, James, and Wetzel, 1986). The Department of Defense is complying with the requirements of the Superfund legislation with the Installation Restoration Program (IRP). The EPA has a backlog of 15,000 sites that may present a groundwater problem, and those are estimated to be less than half the sites in the country that may be contaminated (Brooks and McGinty, 1987). For FY 87, about $1.1 billion was allocated for the EPA to spend on Superfund projects, many of which will entail treating groundwater. The appropriation is part of a $9 billion, five-year program authorized by Congress under SARA, which establishes a preference for remedial actions that permanently and significantly reduce the volume, toxicity, and mobility of hazardous wastes. Estimates indicate that over the next 50 years, approximately $100 billion will be spent on groundwater reclamation programs (Fredrickson and Hicks, 1987). The National Priorities List is a compilation of hazardous waste sites for priority cleanup maintained by EPA (Steelman and Ecker, 1984). After spending more than $1 billion, the Superfund had completed remediation of only six sites on this list (Brown, Loper, and McGarvey, 1986).

The EPA's Risk Reduction Engineering Laboratory has initiated a Chemical Countermeasures Program to define technical criteria for using chemicals and

other additives at contamination sites, such that the combination of the contaminant plus the chemical or other additive, including any resulting reaction products, results in the least overall harm to human health and to the environment (Ellis, Payne, and McNabb, 1985). Under this program, the efficacy of *in situ* treatment of large volumes of subsurface soils, such as found around uncontrolled hazardous waste sites, and treatment of large, relatively quiescent waterbodies contaminated with spills of water-soluble hazardous substances, will be evaluated.

There are other Federal statutes that could be used to protect groundwater, but the above mentioned are the most important (Pye and Patrick, 1983).

State Regulations

States have long been involved with groundwater allocation law and water rights, but it is only in the last decade that they have made large efforts to prevent, abate, and monitor groundwater pollution (Pye and Patrick, 1983). State regulations that may affect groundwater quality fall into three main categories: 1) those dealing with particular sources of pollution, such as septic tank systems and waste disposal sites, 2) those establishing and implementing water quality standards for aquifers, and 3) those regulating the use of land in areas overlying critical aquifer recharge zones.

A waste service industry has emerged in response to the national concern for management of hazardous waste under RCRA (Scholze, Wu, Smith, Bandy, and Basilico, 1986). Biological processes have been employed for treatment of hazardous wastes for many years. One goal of the Superfund remedial program is to promote on-site cleanup of contaminated sites, and the biological techniques are receiving increasing attention as a cost-effective remedial action.

Biotechnology can provide a useful tool to develop new and safer approaches for hazardous waste disposal and cleanup (Nicholas, 1987). However, such developments have been slowed by regulatory uncertainties and the lack of appropriate incentives to promote alternatives to land disposal of hazardous waste. There have been few reports of biotechnology companies entering the bioreclamation field. Most companies will not fund biotechnology research on waste treatment. Much of the research interest and funding have centered on laboratory studies of the use of indigenous microorganisms for waste treatment, in preference to development of a process technology.

1.2 BIODEGRADATION AS A TREATMENT ALTERNATIVE

During the past decade, Government, industry, and the public have recognized the need to greatly reduce the volume and toxicity of waste and to develop safe, effective, and economic alternatives for its disposal (Nicholas, 1987). Problems associated with the cleanup of disposal sites and spills of toxic substances further demonstrate the need for the development of new technologies.

Protection of aquifers from contamination is probably the best solution to the problem, since cleanup of contaminated groundwater is likely to be difficult and expensive (Josephson, 1980). However, once groundwater pollution has occurred, there are a number of techniques that can be used to clean up the contamination (Lee and Ward, 1985; Lee and Ward, 1986; Lee, Wilson, and Ward, 1987). Techniques currently in use include controlling the flow of the polluted groundwater or physical containment of the material; removal of contaminated soil to a secure site; in situ treatment by physical, chemical, or biological techniques; or withdrawal of groundwater, followed by surface treatment by chemical, physical, and biological processes. Often, the volatile or soluble contaminants are sorbed onto activated carbon or resin, which can be very costly.

Traditional methods of treating groundwater contamination have relied upon removal or containment; however, these methods have had limited effectiveness (Brown, Loper, and McGarvey, 1986). Traditional remediation efforts at hazardous waste sites have been partially effective 54 percent of the time and completely successful only 16 percent of the time (Lee and Ward, 1985; Neely, Walsh, Gillespie, and Schauf, 1981). Most treatment schemes currently in use are not completely effective and do not offer permanent solutions for containment or remediation. Some methods may create additional uncontrolled hazardous waste sites.

Removal and/or containment were found to be the most common techniques in a survey of 169 remedial actions (Neely, Walsh, Gillespie, and Schauf, 1981). Removal involves excavation and transport of the contaminated soil to a secure site, such as a landfill. Excavation is usually accomplished by a dragline, which can reach a maximum depth of 60 ft (18.3 m) or a backhoe that can go to 70 ft (21.3 m) (Ehrenfeld and Bass, 1984). Excavation costs between $1.75 to 4.50/ cubic yard ($2.30 to 5.90/ cubic meter; plus additional costs for transportation of the contaminated material and disposal in an approved facility. Total costs are between $200 and 500/cu yd. It may be difficult to excavate all the contaminated soil in instances where contamination is widespread or when the contaminated soil is deep below the land surface. Physical plume containment measures include slurry trench walls, grout curtains, sheet piling, block displacement, infiltration controls, and passive interceptor systems (Lee, Wilson, and Ward, 1987; Canter and Knox, 1984).

There are some circumstances where excavation of contaminated soils is a practical cleanup method (FMC Aquifer Remediation Systems, 1986). For instance, in repairing or replacing underground tanks or pipe lines, contaminated soil must be removed to gain access to the faulty equipment. Excavation can also be practical in immediately dealing with high concentrations that pose a health or environmental hazard. Excavation can also be cost-effective for preventing groundwater contamination from substances limited to the surface.

However, excavation raises the question of what to do with the contaminated soil. It essentially transfers the contamination from one site to another. In addition, soil excavation affects only some of the contaminated soil; it will not treat groundwater and may accentuate groundwater contamination through soil disturbance. Extractions of free contaminant product or contaminated groundwater will not address the material adsorbed on the soil and, therefore, are very long-term or incomplete cleanup methods.

Groundwater contamination by organic chemicals is a multiphase problem (Brown, Loper, and McGarvey, 1986). Organic contaminants in the subsurface can be present as three phases: mobile free product, residually saturated soil (adsorbed phase), and contaminated groundwater (dissolved phase). All three must be addressed to control groundwater quality. Free product must be removed, or it will continue to contaminate the soil through sorption and groundwater movement (vertical and horizontal) and continue to dissolve into the groundwater. If the residually saturated soil is untreated, it can also be a continuing source of contaminated groundwater. The gross contamination must be removed to the point that continued leaching of contamination does not exceed the attenuation capacity of the aquifer (FMC Aquifer Remediation Systems, 1986). Then, the impact of groundwater contamination will have been contained and reversed.

Biological processes have been used both _in situ_, without removal of contaminated groundwater, and on-site (above ground), following removal, to restore contaminated aquifers (Lee and Ward, 1985). _In situ_ and on-site treatment processes avoid the economic and technical disadvantages, as well as environmental risks, incurred by transport of this type of hazardous material to alternate treatment facilities (Ahlert and Kosson, 1983). _In situ_ treatment through chemical or biological transformation of the contaminant has the advantage of dealing with all aspects of the contamination problem and, by destroying the contaminant, of providing a permanent solution (FMC Aquifer Remediation Systems, 1986). Pump-and-treat methods (above ground) treat only the groundwater, while the contaminated soil continues to recontaminate the groundwater. In contrast, _in situ_ treatment can reach organics trapped or sorbed by the soil matrix (Lee and Ward, 1985). _In situ_ treatment is often less expensive than surface treatment or disposal methods and has been especially effective in remediating petroleum and volatile organic spills (FMC Aquifer Remediation Systems, 1986). In many cases, a combination of _in situ_ and on-site treatment will achieve the most cost-effective results at an uncontrolled waste site (Environmental Protection Agency, 1985b). Therefore, both of these approaches will be dealt with in this report. The decision to use any individual procedure or combination of techniques will depend upon the contamination problems at a given site.

Natural attenuation in the subsurface environment is accomplished by biochemical degradation, evaporation, adsorption, metabolism, and transformation by microorganisms (Brown, Loper, and McGarvey, 1986). It is well known that microorganisms are capable of degrading a wide range of organic compounds (Pierce, 1982a).

Figure 1 summarizes the possible fate of xenobiotic compounds in the environment.

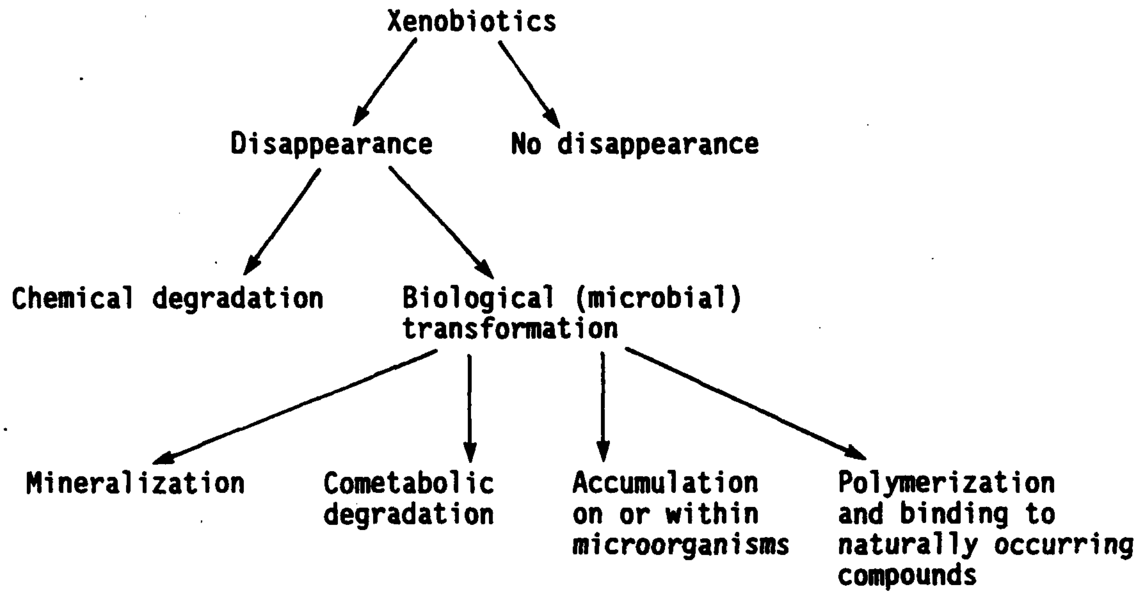

Figure 1. Possible Fate of Xenobiotic Compounds in the Environment (Leisinger, 1983)

An organic chemical may be subjected to nonenzymatic or enzymatic reactions brought about by microorganisms in the subsurface environment (Alexander, 1980); however, it is the enzymatic reactions that bring about the major changes in the chemical structure of these compounds. Extensive removal of organic materials is accomplished primarily through enzymatically mediated biological reactions, i.e., biodegradation (Thornton-Manning, Jones, and Federle, 1987). Few abiotic processes completely mineralize complex organic compounds in soil, and complete degradation depends upon microbial activity (Alexander, 1980). However, physical/chemical transformation processes may act synergistically with biochemical decomposition in this process.

The term "biodegradation" is often used to describe a variety of quite different microbial processes that occur in natural ecosystems, such as mineralization, detoxication, cometabolism, or activation (Alexander, 1980). It can be defined as the breakdown of organic compounds in nature by the action of microorganisms, such as bacteria, actinomycetes, and fungi (Sims and Bass, 1984). The microorganisms derive energy and may increase in biomass from most of the processes (Lee and Ward, 1985).

Mineralization occurs when there is complete biodegradation of an organic molecule to inorganic compounds; i.e., carbon dioxide, water, and mineral ions such as nitrogen, phosphorus, and sulfur (Sims and Bass, 1984; JRB Associates, Inc., 1984b). Under anaerobic conditions, methane may be produced, and nitrate nitrogen may be lost as N_2 or N_2O gas through denitrification. Microorganisms can also transform hazardous organic compounds into innocuous or less toxic organic metabolic products. Chemical alteration can also be the result of cometabolism (cooxidation); i.e., growth on another substrate while the organic molecule is degraded coincidentally (Alexander, 1980). This process may be promoted by enzymes that catalyze reactions of chemically related substrates.

Contaminants in solution in groundwater, as well as vapors in the unsaturated zone, can be completely biodegraded or transformed to new compounds (Wilson, Leach, Henson, and Jones, 1986). Biodegradation converts petroleum components to compounds of lower molecular weight, while biotransforation can convert them to more polar compounds of a carbon number equal to the parent compound (Atlas, 1977). Although the compositions of refined oils, crude oils, and oily wastes are quite different, approaches toward their stimulated biodegradation have been similar.

Organisms that occur naturally in almost every soil system (the indigenous microbial populations) appear to be the chief agents involved in the metabolism of chemicals in waters and soils. Heterotrophic bacteria and fungi are responsible for most of the chemical transformations. Aerobic bacteria, including the actinomycetes and cyanobacteria, anaerobic bacteria, fungi, and some true algae have all been shown capable of degrading many classes of organic chemicals (Amdurer, Fellman, and Abdelhamid, 1985).

Many aerobic bacteria found in soil and water utilize biologically produced substances, such as the sugars, proteins, fats, and hydrocarbons of plant and animal wastes, and can also metabolize petroleum hydrocarbons, converting them to carbon dioxide and water (Brown, Norris, and Brubaker, 1985). Anaerobic bacteria are important for the biodegradation of many chlorinated pesticides and heavily halogenated organics (e.g., trichloroethylene, pentachlorophenol). During biodegradation, certain anaerobic bacteria

(fermenters) commonly produce short-chain organic acids, while other microorganisms further break down these by-products to methane, carbon dioxide, and inorganic substances (Pettyjohn and Hounslow, 1983).

The ability of microorganisms to degrade or transform toxic substances has been applied to the treatment of environmental contamination by hazardous organic compounds. Biodegradation has been used, with reasonable success, to treat aquifers contaminated with petroleum hydrocarbons (Lee and Ward, 1985). Biorestoration is useful for hydrocarbons, especially water-soluble compounds and low levels of other compounds that would be difficult to remove by other means. Biodegradation is environmentally sound, since it destroys organic contaminants and, in most cases, does not generate problem waste products.

Natural soil bacteria may be present in a dormant or slow-growing state, but when stimulated by a specific set of environmental conditions, may multiply rapidly and subsequently adapt to the new environment (Buckingham, 1981). Some of the more common genera of bacteria involved in biodegradation of oil products include Nocardia, Pseudomonas, Acinetobacter, Flavobacterium, Micrococcus, Arthrobacter, and Corynebacterium. Tests have revealed that cultures containing more than one genus appear to have greater hydrocarbon-utilizing capabilities than many of the individual culture isolates.

Degradation in contaminated soil and aquifers may be affected by environmental constraints, such as dissolved oxygen; pH; temperature; toxicants; oxidation-reduction potential; availability of inorganic nutrients, including nitrogen, phosphorus, and others; salinity; and the concentration and nature of the organics. The number and type of organisms present in the environment will also play an important role in this degradation process.

Treatment, therefore, generally consists of optimizing conditions of pH, temperature, soil moisture content, soil oxygen content, and nutrient concentration to stimulate growth of the organisms that will metabolize the particular contaminants present (Sims and Bass, 1984). Optimum environmental conditions and nutrient application rates generally have to be established in laboratory/bench-scale studies and small field pilot tests. Some hazardous compounds may be degraded more readily under aerobic conditions; and some, under anaerobic conditions. Anaerobic conditions are always present at microsites in soil. Treatment might, therefore, consist of a combination of both aerobic and anaerobic conditions.

Biodegradation techniques are versatile and can be used at several different stages of treatment (Nicholas, 1987). Some applications include removal of contaminants from raw materials before processing, treatment of pipeline wastes before discharge from the factory, decontamination of soils and surface and groundwater, and cleanup of dump sites. These operations can be conducted in a number of ways: microorganisms or their active products (e.g., enzymes) can be released directly into the contaminated environment; microorganisms already present in the environment can be enhanced by the addition of suitable nutrients; or microorganisms can be used in contained or semicontained reactors.

If the locally occurring organisms are not effective for the given set of contaminants, the soil can be inoculated with microbial isolates that are specific for those compounds (Buckingham, 1981). Microorganisms can also be

selectively adapted by growth on media containing the target chemicals. They can even be genetically engineered to enhance their ability to degrade these compounds. The use of genetically altered microorganisms may, in the future, expand the range of compounds that can be degraded and/or accelerate the rates at which degradation occurs (Walton and Dobbs, 1980).

Dissolved oxygen may promote biodegradation of wastes, such as aromatics, while some compounds, such as highly chlorinated compounds, can be degraded more readily without oxygen (Lee, Thomas, and Ward, 1985). Studies indicate that oxygen and nutrients are particularly essential to bacterial stimulation in contaminated sites and groundwater (Buckingham, 1981). Therefore, a successful biodegradation scheme requires careful site monitoring of these parameters, through proper sampling and testing procedures. Subsequent oxygen and nutrient management of soil or groundwater, or both, ensures that proper levels are maintained.

Biodegradation is a viable option for treatment of hazardous chemical spills (Buckingham, 1981). Its feasibility depends upon the availability of necessary equipment and manpower, as well as upon decontamination time restrictions. Biodegradation of massive spills may prove ineffective in terms of percent recovery per unit time, whereas it may be the only treatment alternative for low level quantities or concentrations of contaminants.

According to the Office of Technology Assessment (OTA), once developed and proved, biodegradation is potentially less expensive than any other approach to neutralizing toxic wastes (Nicholas, 1987). Such systems involve a low capital investment, have a low energy consumption, and are often self-sustaining operations. Biological means of decomposition require less energy than physico-chemical processes and can be a competitive option under certain circumstances (Ghisalba, 1983).

Biological degradation of constituents in hazardous industrial wastewaters is now being practiced commercially (Scholze, Wu, Smith, Bandy, and Basilico, 1986). For commercial applications of biological technologies to achieve more widespread use, the biotechnologies must meet specific criteria:

1. Degradation

The end result of biological treatment should be to completely destroy the hazardous constituent of concern. This will allow the residues to be disposed of in nonhazardous landfills at a much lower cost.

2. Concentration

Dilute hazardous wastes can be difficult to manage cost effectively, since many chemical and thermal treatments are only cost-effective on concentrated waste constituents. Dilute hazardous wastes might be most cost-effectively treated by using biological treatment to concentrate the organic constituents, followed by thermal treatment of the residue.

3. Diverse Target Constituents

Treatment often has to include management of recalcitrant compounds or combinations of many hazardous constituents. Ideally, organisms in commercial

applications should be able to degrade mixtures of organics, with an equal efficiency for all components. It would also be advantageous for the organisms to accomplish multiple tasks, such as degrading organics while they concentrated inorganics for further treatment or recovery.

4. Consistency

Variability in degradation efficiency among batches would be costly to a waste management firm; consistency in the composition of the residue is essential. The end product of the biological process must be predictable to keep monitoring costs down. Consistency in the residue also allows the biological process to be used in sequence with many solidification treatment processes, which may be sensitive to changes in constituent concentrations.

Also, many solidification treatment processes that could be used in sequence with biological concentration of hazardous constituents, often are sensitive to changes in constituent concentrations.

5. Relatively Low Cost

It has been difficult to reduce the use of landfills because of the high costs associated with alternative options. Biological treatment must be able to compete with other processes (e.g., chemical and thermal applications) in effectiveness and cost.

1.2.1 On-site Biological Treatment Techniques

Conventional biological wastewater treatment techniques that employ microorganisms can be used to treat polluted groundwater (Lee and Ward, 1986). Both aerobic and anaerobic biological treatment systems are available (Roberts, Koff, and Karr, 1988). The systems that have used acclimated bacteria to restore contaminated aquifers typically have relied upon biological wastewater treatment techniques, such as activated sludge, rotating biological contactors, aerated and anaerobic lagoons, composting, waste stabilization ponds, trickling filters, and fluidized-bed reactors for the treatment of relatively large flow volumes with consistent loading characteristics (Lee and Ward, 1985; Lee and Ward, 1986). These processes are described in Appendix A. However, these designs are limited in their scope of application when dealing with groundwater treatment. Contaminated groundwater is characterized by low-flow conditions, varying influent concentrations, decreasing system loading with time, and relatively short-term installation and operating periods.

Many of these systems recharge the effluent from biological treatment to the aquifer to create a closed loop of recovery, treatment, and recharge, which flushes the contaminants out of the soil rapidly and establishes hydrodynamic control to separate the contaminated zone from the rest of the aquifer (Lee and Ward, 1985; Lee and Ward 1986). Acclimated bacteria can also be added to the aquifer and can act in situ to degrade the contaminants. The recharge water can be adjusted to provide optimal conditions for the growth of the acclimated bacteria and of the indigenous populations, which may also act on the contaminants.

1.2.2 In Situ Bioreclamation

In situ treatment of contaminated soil is conducted without excavation and encompasses four methods: Treatment, involving in-ground chemical or biological transformation of contaminants to harmless products; Stabilization, involving the in-ground chemical immobilization of contaminants; Solidification, involving the in-ground physical immobilization of contaminants; and Extraction, involving a subsurface displacement of soil, followed (usually) by chemical treatment on the surface (Amdurer, Fellman, and Abdelhamid, 1985).

In situ biodegradation, commonly referred to as bioreclamation, is based upon the concept of using microflora to decompose the organic compounds contaminating a site to within acceptable levels by treating the soil and groundwater in place (Environmental Protection Agency, 1985ba). Enhanced bioreclamation involves stimulating the indigenous microbial population at the site to improve degradation of the contaminants (Lee and Ward, 1985). The contaminants must not be toxic to the microorganisms that would be available for degradation, and microbes specific for certain chemicals may be encouraged by manipulating environmental conditions.

The bioreclamation method that has received the most developmental effort, and that is the most feasible for in situ treatment, is one that relies on aerobic microbial processes (Environmental Protection Agency, 1985b). This technique provides essential nutrients and oxygen, which may be limiting factors, to stimulate microbial growth (Lee and Ward, 1985). Unpolluted water typically contains very low concentrations of organic and inorganic nutrients and oxygen (Brown, Loper, and McGarvey, 1986). (While unpolluted water would contain oxygen, the 8 to 10 ppm present at saturation is very low.) An organic contaminant introduced into the subsurface provides a source of carbon for the biosynthesis of new cellular material, as long as other constituents of cellular biomass and molecular oxygen are also available.

Fuel contaminants are hydrocarbons and are primarily carbon and hydrogen; cell material is composed of carbon, hydrogen, nitrogen, phosphorus, and oxygen (and minute amounts of other elements). Since most organic contaminants do not contain all the necessary elements for bacterial growth, adequate amounts of oxygen, nitrogen, sulphur, phosphorus, and certain trace minerals must be supplied to enhance the biodegradation rate.

The key to in situ bioreclamation is supplying the needed nutrients and oxygen to the contaminated area for aerobic degradation. Since normal groundwater flow ranges from 10 to 200 ft/yr, it could take years for nutrients to traverse even a small site. By increasing the gradient via pumping (drawdown) and injection (mounding), transport times can be reduced from years to days or even hours. If nutrient availability is limiting the process at a particular site, the more quickly the nutrients can be added, the faster the remediation can begin. In situ treatment generally involves the installation of a bank of injection wells at the head of, or within the plume of, contaminated groundwater (Ward and Lee, 1984).

Anaerobic microorganisms are also capable of degrading certain organic contaminants (Environmental Protection Agency, 1985b). Methanogenic consortia, groups of anaerobes that function under very reducing conditions and produce

methane as an end product, are able to degrade heavily halogenated aliphatics (e.g., perchloroethylene (PCE) or trichloroethylene (TCE)), while aerobic organisms have limited capability. Using anaerobic degradation as an in situ reclamation approach is, theoretically, feasible. However, the process is limited in substrate range and length of time required for degradation.

The following treatment variables or site conditions need to be determined to assess whether degradation could serve as an effective in-place treatment (Lee and Ward, 1986).

1. Biodegradability of the waste constituents in the contaminated soil of interest or similar soil

2. Soil pH

3. Microorganisms present (type, population, genus, species)

4. Soil oxygen content

5. Oxidation-reduction potential

6. Soil moisture

7. Soil nutrient content (C:N:P ratio)

8. Soil temperature

9. Rates of biodegradation

10. Potential for leaching and possible rates of movement of waste constituents through the soil by leaching

11. Soil permeability/porosity

The problem must first be identified and defined. Before any treatment program can be implemented, a thorough investigation of the hydrogeology and contamination problems of the site must be made (Lee, Wilson, and Ward, 1987). Extensive monitoring from a number of monitoring wells is usually required to determine the aquifer characteristics and the extent of contamination. Geophysical techniques, such as ground-penetrating radar, electric resistivity soundings, borehole geophysics, seismic refraction profiling, remote sensing, and magnetometry, may fill the gaps in hydrogeologic information without the costs of additional well installation (Josephson, 1983b). For successful application of in situ technologies, aquifer flow patterns must be determined and channeled to the treatment area (Bove, Lambert, Lin, Sullivan, and Marks, 1984). This may require construction of hydraulic barriers (e.g., slurry walls) to ensure that all groundwater flows to or through the treatment area. The next step is to define the nature and magnitude of potential impacts (FMC Aquifer Remediation Systems, 1986). And the final step is to manage the problem. If there is no impact, this can be simple monitoring and plume management. If there is an impact, some degree of remediation must be undertaken.

Logical steps in the _in situ_ bioreclamation process are 1) site investigation, 2) free product recovery, 3) microbial degradation optimization study, 4) system design, 5) operation, and 6) monitoring (Lee and Ward, 1985). These are described in detail in Appendix A, Section 5.5, Biodegradation Implementation Plan.

SECTION 2

ORGANIC COMPOUNDS IN REFINED OILS AND FUELS

2.1 CHEMICAL COMPOSITION OF FUEL OILS

Most petroleum products can be fractionated into a saturate or aliphatic fraction, an aromatic fraction, and an asphaltic or polar fraction (Brown, Ramos, Fiedman, and Macleod, 1969). Hydrocarbons within the saturated fraction include straight chain alkanes (n-alkanes), branched alkanes, and cycloalkanes (naphthenes) (Atlas, 1981). Since oils and fuels are seldom the same age or from the same source, rarely do they have the same chemical composition. Fuel oils of similar viscosity and volatility may vary widely in composition, dependent upon the source of the raw materials or method of manufacture (Naval Civil Engineering Laboratory, 1986). Due to chromatographic column inefficiency, a gas chromatogram of an oil may actually reveal fewer than one-tenth of 1 percent of all the components present in a sample (Blumer, 1975). Thus, there may be many more chemical compounds present in a sample than can be detected, and it is impossible to assign a standard composition to the different oils and fuels. However, to the extent possible, knowing what compounds are present in a contaminated site will help with correlating the degradative capabilities of different microorganisms for greater efficiency in biodegradation.

2.1.1 Naptha

Petroleum naphtha is a name sometimes given to a portion of the more volatile hydrocarbons distilled from petroleum. Naphtha is a generic term for a group of refined, partly refined, or unrefined petroleum products (primarily gasoline) in which not less than 10 percent of the product distills below 347°F (175°C) and not less than 95 percent distills below 464°F (240°C) (Holmes and Thompson, 1982). It forms highly complex mixtures of hydrocarbons that range from paraffin-type hydrocarbons to aromatics, which can be difficult to classify based upon chemical properties (Bland and Davidson, 1967).

2.1.2 Kerosene

Kerosene applies to that portion of petroleum boiling between the approximate limits of 180° and 320°C. It can be used as jet fuel. The chemical composition of kerosene depends upon its source and usually consists of a mixture of about 10 hydrocarbons containing 10 to 16 carbon atoms/molecule. The constituents include n-dodecane, alkyl derivatives of benzene, naphthalene, and derivatives of naphthalene.

2.1.3 Fuel Oil and Diesel #2

Diesel fuel is the fraction of petroleum that distills after kerosene (396° to 583°F or 202° to 306°C) (Holmes and Thompson, 1982). Other than for nitrogen content, the shale- and petroleum-derived diesel fuels are comparable (Ghassemi and Panahloo, 1984). The organic components of Diesel fuel #2 can be found in Appendix A, Section 2.1.3.

2.1.4 Gasoline

Gasoline is composed of several hundred different hydrocarbon compounds in various proportions, primarily C_5 to C_9 hydrocarbon chain fractions with a boiling range of 80^0 to 400^0F (23^0 to 204^0C) (PEDCO Environmental, Inc., 1978). The basic types of hydrocarbons in gasoline are paraffins, olefins, cycloparaffins, and aromatics. There will also be some sulfur, nitrogen, and oxygen present and trace amounts of iron (0.07 to 13 ug/ml), nickel (0.001 to 2 ug/ml), and zinc (0.001 to 2 ug/ml) (Ghassemi and Panahloo, 1984). Appendix A provides a breakdown of the composition of gasolines.

2.1.5 JP-5

JP-5, "jet fuel," is made by blending naphtha, gasoline, and kerosene to meet military and commercial product standards (Ghassemi and Panahloo, 1984). The content of its highly toxic components varies widely among different jet fuels. One petroleum-derived JP-5 sample had a concentration of 0.04 ug/g of benzo(a)pyrene, while shale oil-derived JP-5 contained none. JP-5 can contain high molecular weight normal paraffins or disulfides, and if it is prepared by hydrocracking, it will have a considerable degree of branching (Varga, Lieberman, and Avella, 1985). See Appendix A, Section 2.1.5, for the organic compounds and trace elements present in JP-5.

2.1.6 JP-4

JP-4, "jet fuel," is made by blending various proportions of naphtha, gasoline, and kerosene to meet military and commercial product specifications (Gary and Hardwork, 1975). The content of highly toxic components in this fuel also varies. It has the highest hydrogen content, the highest volatility, and the lowest viscosity of any fuel widely used by the Air Force (General Electric Co. Aircraft Engine Business Gp., 1982). The organic compounds and trace elements present in JP-4 are listed in Appendix A.

2.2 FACTORS AFFECTING CONTAMINATION AND BIODEGRADATION

Crude petroleum and many of the products refined from petroleum contain thousands of hydrocarbons and related compounds (Cooney, Silver, and Beck, 1985). When such petrochemicals enter an aquatic system, they are subject to a number of physical, chemical, and biological factors that contribute to loss or alteration of some of the components. Volatile compounds can be selectively lost by evaporation; photochemical reactions may contribute to change; the oil may adsorb to detritus; wind, wave, or other mixing actions may cause formation of emulsions; and microorganisms can metabolize and cometabolize most of the hydrocarbons under proper environmental conditions.

The fate of a pollutant in the environment is determined by characteristics of both the chemical and the environment (Pfaender, Shimp, Palumbo, and Bartholomew, 1985). Significant waste factors that will affect its biodegradation include (Parr, Sikora, and Burge, 1983):

1. Chemical composition of the waste
2. Its physical state (i.e., liquid, slurry, sludge)
3. Its carbon:nitrogen ratio
4. Water content and solubility in water
5. Chemical reactivity with and dissolution effects on soil organic matter
6. Volatility
7. pH
8. Biochemical oxygen demand (BOD)
9. Chemical oxygen demand (COD).

The behavior of toxic pollutants in the environment also depends upon a variety of processes and properties (Ghisalba, 1983; Josephson, 1983; Bitton and Gerba, 1985; Pettyjohn and Hounslow, 1983).

1. Chemical processes, e.g.:

   hydrolysis
   photolysis
   oxidation
   reduction
   hydration

2. Physical or transport processes and properties, e.g.:

   advection
   dispersion and diffusion
   sorption
   volatilization

solubilization
viscosity
density
dilution

3. Biological processes, e.g.:

   bioaccumulation
   biotransformation
   biodegradation
   toxicity

4. Combined environmental factors.

There are major compositional variations between different crude and refined oils (Atlas, 1978c). Some oils contain toxic hydrocarbons, which may prevent or delay microbial attack; some refined oils have additives, such as lead, which can inhibit microbial degradation of polluting hydrocarbons. Oils contain varying proportions of paraffinic, aromatic, and asphaltic hydrocarbons. Both the rate and extent of biodegradation are dependent upon the relative proportions of these classes of hydrocarbons.

Under favorable conditions, microorganisms will degrade 30 to 50 percent of a crude oil residue, but degradation of such a residue is never complete and does not affect the different main hydrocarbon groups in the same manner (Solanas and Pares, 1984). Many synthetic organic compounds (Pettyjohn and Hounslow, 1983), and many complex hydrocarbon structures in petroleum (Atlas, 1978c), are not easily broken down by microbial action. However, with favorable conditions and the proper organisms, virtually all kinds of hydrocarbons--straight chain, branched chain, cyclic, simple aromatic, polynuclear aromatic, asphaltic--have been found to eventually undergo partial or complete oxidation (Texas Research Institute, Inc., 1982).

Each organic compound has unique characteristics that dictate which of the above mechanisms or combination of mechanisms controls its movement and degradability (Josephson, 1983b). Appendix A, Section 2.2, discusses in depth the role of these factors and processes in biodegradation.

2.2.1 Physical, Chemical, Biological, and Environmental Factors

Biodegradation can be affected by physical, chemical, biological, and environmental factors, such as solubility, advection, dispersion and diffusion, sorption, volatility, viscosity, density, chemical structure, environmental factors, toxicity, concentration, naturally occurring organic materials, hydrolysis and oxidation, and biological factors. These are discussed in depth in Appendix A, Section 2.2.1.

2.2.2 Rate of Biodegradation

It is important to have information on the rates of pollutant biodegradation under environmental conditions to be able to assess the potential fate of the compounds (Pfaender and Klump, 1981). The rate of mineralization of a number of organic compounds is directly proportional to their concentration over a wide range of concentrations (Alexander, 1985).

The parameters influencing the rate of biodegradation are of two types (Buckingham, 1981):

1. Those that determine the availability and concentration of the compound to be degraded or that affect the microbial population site and activity

2. Those that control the reaction rate.

The kinetics of biodegradation are zero-order, if the concentration is high relative to microbes that can degrade it, or first-order, if the concentration is not high enough to saturate the ability of the microbes (Kaufman, 1983). First-order kinetics apply where the concentration of the chemical being degraded is low relative to the biological activity in the soil; i.e., at any one time, the rate of chemical loss is proportional to its concentration in the soil (Hill, McGrahen, Baker, Finnerty, and Bingeman, 1955; Kearney, Nash, and Isensee, 1969). Michaelis-Menten kinetics seem to apply when the chemical concentration increases and the rate of decomposition changes from being proportional to being independent of concentration (Hamaker, 1966).

In most natural ecosystems, the numbers of hydrocarbon-utilizing microorganisms present will initially limit the rate of hydrocarbon degradation (Atlas, 1978c). But after a short period of exposure to petroleum pollutants, the numbers of hydrocarbon utilizers increase and will no longer be the principal rate-limiting factor.

In natural ecosystems, a variety of factors probably alter the shapes of substrate disappearance curves (Alexander, 1986). These factors may include predation by protozoa, the time for induction of the active organisms, the accumulation of toxins produced by other microorganisms, depletion of inorganic nutrients or growth factors, the presence of other substrates that may repress utilization of the compound of interest, and binding of the compound to colloidal matter. The impacts or interactions of such potentially important factors may make it difficult to predict the kinetics of mineralization or disappearance of a particular substrate.

The rates of degradation of long chain alkanes will depend upon the availability of the hydrocarbon to microorganisms (Atlas, 1978c). Availability will be greatly restricted by very low solubility and low surface area of contaminant to microbe for long chain alkanes, which are solid at normal environmental temperatures. Extensive branching also tends to reduce rates of hydrocarbon degradation.

Other factors that appear to have a major influence on rates of biodegradation include soil temperature and salinity (Palumbo, Pfaender, Paerl, Bland, Boyd, and Cooper, 1983).

There is considerable variation in the rate of biodegradation of specific contaminants that enter the subsurface environment (Wilson, Leach, Henson, and Jones, 1986). Rates can vary two to three orders of magnitude between aquifers or over a vertical separation of only a few feet in the same aquifer. Different biotransformation rates can be found for the same organic chemical tested by varying some parameter in the biotransformation feasibility testing (Leslie, Page, Reinert, Moses, Rodgers, Dickson, and Hinman, 1983). The following

factors have to be considered when conducting biotransformation experiments: sediment concentration, sediment organic content, and amount of oxygen available to microorganisms in the environment.

Sections 2.2.2 and 2.2.3 in Appendix A further discuss the kinetics of biodegradation and provide the degradation rates for a number of organic chemicals.

2.2.3 Effect of Hydrocarbon Concentrations on Degradation

The concentration of a hydrocarbon can affect its biodegradability and toxicity to the degrading organisms (Environmental Protection Agency, 1985b). High concentrations of a hydrocarbon can be inhibitory to microorganisms, and the concentration at which inhibition occurs will vary with the compound (Alexander, 1985). Concentrations of hydrocarbons in the range of 1 to 100 ug/ml of water or 1 to 100 ug/g of soil or sediment (on a dry weight basis) are not generally considered to be toxic to common heterotrophic bacteria and fungi.

In some cases, relatively high concentrations of a pollutant (>100 ug/l) can stimulate multiplication of the microbes that metabolize the organic contaminant (Wilson and McNabb, 1983). However, concentrations of less than 10 ug/l of the material usually do not have this effect. It has been reported that if groundwater is taken from an anaerobic gasoline- or fuel oil-contaminated area with no significant degradation activity, and oxygen and nutrients are then added, rapid degradation of the hydrocarbons down to 1 ug/l or less starts with little lag time (Jensen, Arvin, and Gundersen, 1985). This requires an initial hydrocarbon concentration below about 10 mg/l. At higher concentrations, a considerable adaptation time may be expected. If the concentrations are greater than 1 to 10 mg/l, metabolism of the compound can entirely deplete the oxygen or other metabolic requirements available in the groundwater (Wilson and McNabb, 1983).

Appendix A, Section 2.2.3, provides examples of concentrations of specific organic compounds that microorganisms have been able to tolerate and degrade. Both initial and final concentrations are given for target chemicals at contaminated sites treated with biodegradation. The effect of low concentrations of chemicals on biodegradation is also discussed in Appendix A.

SECTION 3

INDIGENOUS MICROORGANISMS IN BIODEGRADATION

Microorganisms are the principal agents responsible for the recycling of carbon in nature. In many ecosystems there is already an adequate indigenous hydrocarbonoclastic microbial community capable of extensive oil biodegradation, provided that environmental conditions are favorable for oil-degrading metabolic activity (Atlas, 1977). This has been shown for many soil and marine and fresh-water environments (Atlas and Bartha, 1973c; Mironov, 1970; Cooney and Summers, 1976; Cooney, 1974; Mulkins-Phillips and Stewart, 1974a; Litchfield and Clark, 1973). It is suggested by some researchers (Atlas, 1977; McGill, 1977) that all soils, except those that are very acidic, contain the organisms capable of degrading oil products, that microbial seeding is not necessary, and that the problem is actually one of supplying the necessary nutrients at the site.

The ability to utilize hydrocarbons is widely distributed among diverse microbial populations (Atlas, 1981). Many species of bacteria, cyanobacteria, filamentous fungi, and yeasts coexist in natural ecosystems and may act independently or in combination to metabolize aromatic hydrocarbons (Cerniglia, 1984; Fedorak, Semple, and Westlake, 1984; Gibson, 1982). In general, population levels of hydrocarbon utilizers and their proportions within the microbial community appear to be a sensitive index of environmental exposure to hydrocarbons (Atlas, 1981). In unpolluted ecosystems, hydrocarbon utilizers generally constitute less than 0.1 percent of the microbial community; in oil-polluted ecosystems, they can constitute up to 100 percent of the viable microorganisms. This difference seems to quantitatively reflect the degree or extent of exposure of an ecosystem to hydrocarbon contaminants. Extensive degradation of petroleum pollutants generally is accomplished by mixed microbial populations, rather than single microbial species (Atlas, 1978c).

There are advantages to relying on indigenous microorganisms rather than adding microorganisms to degrade wastes (Environmental Protection Agency, 1985b). Through countless generations of evolution, natural populations have developed that are ideally suited for survival and proliferation in that environment. This is particularly true of uncontrolled hazardous waste sites where microorganisms have been exposed to the wastes for years or even decades.

3.1 MICROORGANISMS IN SOIL

Current evidence suggests that in aquatic and terrestrial environments, microorganisms are the chief agents of biodegradation of environmentally important molecules (Alexander, 1980). In 1946, ZoBell (Texas Reserach Institute, Inc., 1982) reported that nearly 100 species of bacteria, yeasts, and molds, representing 30 microbial genera had been discovered to have hydrocarbon-oxidizing properties. Since that time, many other species and genera have been reported to have this ability (Texas Reserach Institute, Inc., 1982) and to be widely distributed in soils (Blakebrough, 1978; Atlas, 1981). Although many microorganisms appear limited to degradation of a specific group of chemicals, others have demonstrated a wide diversification of substrates that they are capable of metabolizing (105).

The metabolic diversity of microorganisms in the soil is an important factor in the biodegradation of xenobiotic or hazardous materials (JRB Associates, Inc., 1984b). Bacteria may be classified metabolically as heterotrophic, if they derive their energy and carbon for survival and growth from the decomposition of organic materials, or as autotrophic, if they fix the carbon they need for growth from carbon dioxide and obtain (usually) their energy from light (photosynthetic or phototrophic) or the oxidation of inorganic compounds (chemosynthetic or lithotrophic). Lithotrophic organisms may transform inorganic materials (especially nitrogen) into available nutrients for the heterotrophs (Focht and Verstraete, 1977; Painter, 1977). Lithotrophic or photosynthetic bacteria may also directly or indirectly transform toxic metals or metalloids.

In soil taken from the surface to 100 ft, facultative oligotrophic bacteria were dominant; these organisms can survive on low levels of nutrients, if necessary (Benoit, Novak, Goldsmith, and Chadduck, 1985). Obligate oligotrophic bacteria, which require low concentrations of nutrients, are the most frequent isolates from deep soil profiles (>100 ft), and yeasts have been present in some samples. Obligate anaerobic bacteria and microaerophilic bacteria have been recovered from deep soil profiles; however, they are a small portion of the population.

The heterotrophic bacteria are the most important organisms in the transformation of organic hazardous compounds, and soil treatment schemes may be directed toward enhancing their activity (JRB Associates, Inc., 1984b). Heterotrophs can use the organic contaminants as sources of both carbon and energy (Knox, Canter, Kincannon, Stover, and Ward, 1968). Some organic material is oxidized for energy while the rest is used as building blocks for cellular synthesis. There are three methods by which heterotrophic microorganisms can obtain energy: fermentation, aerobic respiration, and anaerobic respiration. These processes are discussed in Section 4 and Appendix A.

Some compounds appear to be degraded only under aerobic conditions, others only under anaerobic conditions, and some under either condition, while others are not transformed at all. It has been concluded that hydrocarbons are subject to both aerobic and anaerobic oxidation (Dietz, 1980).

Stratification of microbes results from natural selection in response to chemical and physical gradients in the soil column (Ahlert and Kosson, 1983). Oxygen concentration is a principal gradient. It is greatest at the soil

surface, where diffusion from the ambient air drives the gradient. The concentration of oxygen diminishes with depth and is depleted by aerobic respiration. This produces an aerobic community near the soil surface and anaerobic communities that dominate at greater depths. Other gradients are caused by available nutrients, temperature, and available organic carbon. At all levels in the soil, substrate is consumed and metabolites produced, which may then serve as substrate for other organisms at other locations in the column. As the environmental effects and constituents of waste vary, natural selection and adaptation occur. The biological community is continuously responding to all these dynamic variables.

Table A.3-1 lists a number of organisms and the compounds they are able to degrade.

Table A.3-2 shows the general groups of microorganisms that might be useful for treating contamination by specific types of organic compounds and the conditions that would be most favorable for their development (Kobayashi and Rittmann, 1982).

3.1.1 Bacteria

Numerous bacterial strains and species have been isolated that can degrade many of the petroleum-derived compounds of the greatest hazard to the environment and to health (Nicholas, 1987). The compounds that can be attacked include most of those of major environmental concern, including benzene, toluene, biphenyls, and naphthalene.

Bacteria are predominantly involved with degradation of those chemicals that have a higher degree of water solubility and are not strongly adsorbed (105). The binary fission-type reproductive methods of bacteria enable them to compete more successfully than fungi for readily available substrates. See Appendix A for a description of the small bacterial cells that are found in the subsurface.

3.1.1.1 Aerobes

Aerobic bacteria ultimately decompose most organic compounds into carbon dioxide, water, and mineral matter, such as sulfate, nitrate, and other inorganic compounds (Pettyjohn and Hounslow, 1983), and do not produce hydrogen sulfide or methane as reaction products (Amdurer, Fellman, and Abdelhamid, 1985).

The most commonly isolated organisms in areas of hydrocarbon contamination are heterotrophic bacteria of the genera Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Vibrio, Acinetobacter, Brevibacterium, Corynebacterium, Flavobacterium (Kobayashi and Rittmann, 1982), Mycobacterium (Gholson, Guire, and Friede, 1972; Soli, 1973), and Nocardia (Canter and Knox, 1984). Pseudomonas species appear to be the most ubiquitous and the most adaptable to the different pollutants, while Corynebacterium species may be major agents for decomposing heterocyclic compounds and hydrocarbons in contaminated aquatic environments (Kobayashi and Rittmann, 1982). Appendix A discusses hydrocarbon degradation by some of the more frequently encountered aerobic bacteria.

3.1.1.2 Anaerobes

Anaerobic decomposition of organic matter to carbon dioxide and methane involves interactions within consortia of obligate anaerobic bacteria (Kobayashi and Rittmann, 1982). At least four interacting trophic groups of bacteria are involved:

1. Hydrolytic bacteria that catabolize the major components of biomass, such as saccharides, proteins, and lipids

2. Hydrogen-producing, acetogenic bacteria that catabolize products from the activity of the first group, such as fatty acids and neutral end products

3. Homoacetogenic bacteria that catabolize multicarbon compounds to acetic acid

4. Methanogenic bacteria (Zeikus, 1980)

Obligate anaerobes require not only anoxic (oxygen-free) conditions, but also oxidation-reduction potentials of less than -0.2 V (Kobayashi and Rittmann, 1982). While many soil bacteria can grow under anaerobic conditions, most fungi and actinomycetes cannot grow at all (Parr, Sikora, and Burge, 1983). Anaerobic decomposition is performed mainly by bacteria utilizing either an anaerobic respiration or interactive fermentation/methanogenic type of metabolism. These processes are described in Appendix A, Section 4.1.2. The end products of anaerobic degradation are reduced compounds, some of which are toxic to microorganisms and plants. Table A.4-7 shows the end products formed from degradation of specific organic hydrocarbons by different microorganisms.

3.1.1.3 Oligotrophs

Organisms living at organic concentrations <15 mg carbon/l are termed oligotrophs (Stetzenbach, Sinclair, and Kelley, 1983). They may be able to live under conditions of even lower carbon flux (<1 mg/l/day) (Poindexter, 1981). They do not constitute a special taxonomic grouping of organisms, but come from almost any group of bacteria or chemotrophs. They are generally adapted to life under low nutrient conditions, but can readily be readapted to high nutrient conditions. Reverse adaptation to the low nutrient environment is, however, not readily achieved; therefore, oligotrophs are obtained only from low-nutrient environments.

Oligotrophs generally have a high surface/volume ratio and high affinity for substrate (Kobayashi and Rittmann, 1982). The minimum substrate concentration needed for measurable growth is lower than that required for eutrophic (high nutrient) organisms, but the maximum growth rate is also lower. Oligotrophs degrade xenobiotics more slowly than natural compounds (Alexander, 1985); however, their capability of surviving on low concentrations makes them potentially useful for removal of trace concentrations of organic contaminants from water, or effluent from wastewater treatment processes (Poindexter, 1981). Species of <u>Pseudomonas</u>, <u>Flavobacterium</u>, <u>Acinetobacter</u>, <u>Aeromonas</u>, <u>Moraxella</u>, <u>Alcaligenes</u>, and <u>Actinomyces</u> have been detected in water samples, with isolates surviving extended periods on low nutrient concentrations (Stetzenbach, Sin-

clair, and Kelley, 1983). These organisms are usually found living as biofilms (Poindexter, 1981). An important characteristic is that they often appear to have multiple inducible enzymes, are able to shift metabolic pathways, and can take up and use mixed substrates (e.g., a Clostridium sp.).

Some Actinomycetes (Nocardia) coryneforms, and mycobacteria have survived for 30 days under starvation conditions (138). Oligotrophic bacteria from surface water and those indigenous to the deeper subsurface fail to grow on complex media (Wilson, McNabb, Wilson, and Noonan, 1983). Apparently, many oligotrophic bacteria from surface water fail to use organic compounds that are used readily by eutrophic forms. There is an indication that the bacteria of the deeper subsurface will be active against a more limited range of organic compounds than are degraded in surface soil.

Microorganisms appear to have a threshold level below which some organic compounds cannot be converted to carbon dioxide (Alexander, 1985). This level is needed for growth, enzyme induction, and enzyme activity. At lower concentrations, substrate uptake by diffusion of the molecules will meet maintenance energy and survival requirements but will not support growth (Schmidt and Alexander, 1985).

It is not certain whether the trace amounts present in a contaminated site would be sufficient to allow microbial growth or propagation to numbers necessary for biodegradation of the material (Alexander, 1985). If not, this could explain the persistence of low levels of biodegradable organic substances, e.g., toluene, xylenes, naphthalene, and phthalate esters, in water or soil in nature (although this might also be due to oxygen limitation). It should be noted that if these organisms obtain energy and carbon for growth by using natural organic constituents of the environment, the threshold for a particular chemical contaminant may be below the level of detection possible using current analytical procedures.

Some organic compounds are mineralized even at trace levels below 1.0 pg/ml (Alexander, 1985). It is important to distinguish between compounds that can be transformed at low concentrations by large populations of nongrowing cells and substrates that must support growth for significant degradation to occur.

3.1.1.4 Counts in Uncontaminated Soil

Microorganisms are widely distributed in nature, but reports of the actual numbers present is confusing because of the methodological differences used to enumerate the microbes (Atlas, 1981). No place has been found in the U.S. or Canada--at depths to 400 ft--where sufficient organisms are not present to be brought up in 72 hr to a significant population (Rich, Bluestone, and Cannon, 1986). The bacteria are present; the problem is establishing the right conditions for their growth.

Early studies indicated that the number and activity of microbes decline dramatically with increasing depth (see Table A.3-3) (Markovetz, 1971). Recent reports indicate that active microbial populations can be found in deep, unsaturated and saturated subsoils (Pirnik, 1977). These counts show variation with depth when viable but not direct counts are used (Table A.3-4). Aseptic core samples from depths of 300 m (nearly 1000 ft) indicate an abundant and diverse

population of microorganisms extending to well below surface soils and shallow aquifers (Fredrickson and Hicks, 1987).

It appears that different soil types vary in the distribution of biomass and enzymatic activity through their vertical profile (Federle, Dobbins, Thornton-Manning, and Jones, 1986). Biomass and activity are significantly correlated with each other and negatively correlated with depth. While biomass and activity decrease with increasing soil depth, the magnitude of decline differs for different soils. It is difficult to generalize on the level of biomass or activity to expect in a soil based on depth or horizon alone. Soil type is also important in determining the types of microbial populations present. Depth may be responsible for as much as 75 percent of the variation in biomass, but an additional 11 percent of the variation could be explained by pH and silt, clay, and organic contents. Depth also explained 78 percent of the variation in microbial activity; silt content explained another 4.5 percent.

Enumeration of microorganisms can be difficult, since most subsurface bacteria exist in an ecosystem low in organic carbon and do not grow well, if at all, in conventional growth media with high organic carbon concentrations (Wilson, Leach, Henson, and Jones, 1986). In addition, many organisms attach firmly to particles (Federle, Dobbins, Thornton-Manning, and Jones, 1986). Dilution plating techniques yield 1 to 10 percent of the number of cells determined by microscopic direct counting. An alternate approach is to determine biomass by analyzing the phospholipids extractable from soil. This method has been used to estimate microbial biomass in estuarine and marine environments (Gillan, 1983; White, 1983) and in subsurface soils. Appendix D describes many of the traditional and alternative methods for enumerating microorganisms in environmental samples.

It is important to be able to distinguish between viable and nonviable cells. However, it is believed that many organisms in the subsurface will be in a dormant state until stimulated by an appropriate concentration of a suitable substrate (Alexander, 1977). The deeper the soil, the more oligotrophic the organisms will become and, hence, the more fastidious their requirement for low nutrient concentrations. The proportion of hydrocarbon-degrading organisms to total heterotrophs is now considered to be a more significant indicator of the biological activity in the subsurface, with respect to hydrocarbon contaminants.

Direct counts tend to be fairly consistent with soil depth and sampling site and were found to be on the order of 10^6 to 10^7 bacteria/g dry wt of soil (see Appendix A). Plate counts, on the other hand, suggested highly variable and decreasing counts with depth, ranging from zero to 10^8.

3.1.1.5 <u>Counts in Contaminated Soil</u>

Microbial numbers and activity are initially depressed by even light hydrocarbon contamination (Odu, 1972). However, this is followed by a stimulation of activity. The number of hydrocarbon-utilizing organisms in a soil reflects the soil's past exposure to hydrocarbons (Atlas, 1981). These organisms are most abundant in places that have been chronically exposed to hydrocarbon pollution (Texas Research Institute, Inc., 1982). Few or none are found in unpolluted groundwater or petroleum directly from wells. Substantial

adapted populations exist in contaminated zones, with the bacterial biomass increasing as the organic contaminants are metabolized (Environmental Protection Agency, 1985b).

Bacterial counts were 100 to 1,000 times higher inside than outside a zone of contamination of an aquifer containing JP-5 jet fuel (Ehrlich, Schroeder, and Martin, 1985). It appears that only a few species of specialized bacteria, presumably those able to assimilate the hydrocarbons, are preferentially selected for in a contaminated zone.

Appendix A discusses counts found in contaminated soil. Direct counts range from 10^3 to 10^8 organisms/g, while viable counts have been recorded from less than 100 to 10^5 CFU/g. Hydrocarbon degraders have been measured at naturally occurring levels of 10^2 to 10^5 organisms/g. These numbers increase after biostimulation of the contaminated sites (see Section 5.1.1.5).

3.1.2 Fungi

Fungi are eukaryotic microorganisms that lack photosynthetic structures and depend upon heterotrophic metabolism (Solanas and Pares, 1984). They may be filamentous or unicellular (yeast-like or amoeboid), or aggregate to form large structures, such as a plasmodium or fruit-bodies (mushrooms). Fungi are a large part of the microbial biomass in soil, especially in acidic conditions, and they contribute to most decomposition processes. When substrate or water availability is low, most of the fungal biomass is either dormant or dead. Fungal spores or other resistant structures can survive under adverse conditions for long periods of time and then quickly germinate and grow when environmental conditions become favorable. The species and diversity of fungi are affected by clay mineralogy, temperature, and other soil environmental conditions. Most filamentous fungi are aerobic, and yeasts are often facultatively anaerobic. The species of fungi that develop on plates are frequently those that produce spores in greatest abundance (Nannipieri, 1984). Additional colonies can, thus, grow from inactive spores or conidia and give an inaccurate estimate of population size.

Most soil fungi are mesophiles with temperature optima between 25° and $35^{\circ}C$, but with an ability to grow from about 15° to $45^{\circ}C$ (Cooke and Rayner, 1984). Even thermophilic fungi do not grow above about $65^{\circ}C$ (JRB Associates, Inc., 1984b). However, many fungi grow at temperatures below $10^{\circ}C$. The influence of soil acidity on fungal growth is difficult to assess because of the ability of fungi to radically alter the pH of their environment (Cooke and Rayner, 1984). This capability indicates that acidifying soil will not improve growth of the organisms, but does reduce competition from other organisms (Kirk, Schultz, Connors, Lorenz, and Zeikus, 1978).

Fungi play an important role in the hydrocarbon-oxidizing activities of the soil (Jones and Eddington, 1968). They seem to be at least as versatile as bacteria in metabolizing aromatics (Fewson, 1981). Their extracellular enzymes may help to provide substrates for bacteria, as well as for themselves, by hydrolyzing polymers. They are also important sources of secondary metabolites.

Some filamentous fungi, unlike other microorganisms that attack aromatic hydrocarbons, use hydroxylations as a prelude to detoxification rather than

catabolism and assimilation (Dagley, 1981). These organisms do not degrade aromatic hydrocarbons as nutrients, but simply detoxify them. Several fungi (Penicillium and Cunninghamella) even exhibit greater hydrocarbon biodegradation than bacteria (Flavobacterium, Brevibacterium, and Arthrobacter) (154). The ability to utilize hydrocarbons occurs mainly in two orders, the Mucorales and the Moniliales (Nyns, Auquiere, and Wiaux, 1968). Aspergillus and Penicillium are rich in hydrocarbon-assimilating strains. It has been concluded that the property of assimilating hydrocarbons is a property of individual strains and not necessarily a characteristic of particular species or related taxa.

The genera most frequently isolated from soils are those producing abundant small conidia; e.g., Penicillium and Verticillium spp. (Davies and Westlake, 1979). Oil-degrading strains of Beauveria bassiana, Mortieriella spp., Phoma spp., Scolecobasidium obovatum, and Tolypocladium inflatum have also been isolated. Fifty-six out of 500 yeasts studied were found to be able to degrade hydrocarbons; almost all of these were in the genus Candida (Komagata, Nakase, and Katsu, 1964). Hydrocarbonoclastic strains of Candida, Rhodosporidium, Rhodotorula, Saccharomyces, Sporobolomyces, and Trichosporon have been identified from soil (Ahearn, Meyers, and Standard, 1971; Cook, Massey, and Ahearn, 1973). Cladosporium resinae has been found in soil (Walker, Cofone, and Cooney, 1973) and has repeatedly been recovered as a contaminant of jet fuels (Atlas, 1981). This organism can grow on petroleum hydrocarbons and creates problems in the aircraft industry by clogging fuel lines. Specific fungi and some of the hydrocarbons they are able to degrade are discussed in Appendix A.

Bacteria and yeasts show decreasing ability to degrade alkanes with increasing chain length (Walker, Austin, and Colwell, 1975). Filamentous fungi do not exhibit preferential degradation for particular chain lengths and appear to be better able to degrade or transform hydrocarbons of complex structure or long chain length. Because they have nonspecific enzyme systems for aromatic structures, fungi (yeasts and filamentous) are believed to be capable of biodegrading PCBs better than bacteria can (Gibson, 1978). However, fungal metabolism often results in incomplete degradation that necessitates bacterial association for complete mineralization. Whereas bacteria oxidize aromatic hydrocarbons to cis-dihydrodiols, fungi convert them to trans-diols, with arene oxides (epoxides) as intermediates (Dagley, 1981). This suggests that fungi metabolize aromatic hydrocarbons in a manner similar to mammalian systems, i.e., via a monooxygenase-catalyzed reaction (Cerniglia, Hebert, Szaniszlo, and Gibson, 1978). It is probable that a cytochrome P-450 dependent reaction may be responsible for the initial oxygenation of naphthalene by these organisms. The products of fungal metabolism are often recognized carcinogens, a point that supports combining the fungi with bacteria for complete degradation.

Fungi appear to be predominantly involved in metabolizing those xenobiotics of lower water solubility and greater adsorptivity (Kaufman, 1983). The mycelial-type growth characteristics of fungi perhaps enables them to encapsulate and penetrate soil particles to which xenobiotics may be adsorbed. Soil fungi are generally believed to play a more important role in the formation, metabolism, and interactions of soil organic matter complexes than bacteria. This may allow them to better cope with the various bonding mechanisms involved with adsorbed materials.

Table A.3-2 shows the most favorable conditions for growth of hydrocarbonoclastic fungi for removal of contaminants.

3.1.3 Photosynthetic Microorganisms

The surface soil usually supports large populations of eukaryotic algae and cyanobacteria (blue-green algae) (JRB Associates, Inc., 1984b). Since light cannot penetrate far into the soil, the algal biomass is usually low. These organisms may enhance photodecomposition of hazardous organic compounds at the soil surface.

The photosynthetic microorganisms of interest are the algae, cyanobacteria (blue-green algae), and photosynthetic bacteria (Kobayashi and Rittmann, 1982). These organisms are potentiallly importany in situations involving low concentration of nutrients, because they are able to obtain energy from sunlight and carbon by carbon dioxide fixation. Some of the cyanobacteria and photosynthetic bacteria are also able to fix nitrogen; hence, they can survive in situations in which the dissolved nitrogen concentration is inadequate to support bacterial growth.

The ability to oxidize aromatic hydrocarbons is widely distributed among the cyanobacteria and algae. Cyanobacteria, Chlorella (green algae), and especially the Chromatiaceae (photosynthetic bacteria) are pollution tolerant (Kobayashi and Rittmann, 1982; Pfennig, 1978a). Of the latter, the purple sulfur bacteria (Thiorhodaceae) are important. The purple nonsulfur (Rhodospirilliaceae) bacteria are also of interest. For these bacteria, organic compounds serve as the major source of electrons and carbon for cellular components (Berry, Francis, and Bollag, 1987). The photosynthetic bacteria are known to be able to metabolize a wide variety of substances (e.g., simple sugars, alcohols, volatile fatty acids, tricarboxylic acid cycle intermediates, aromatic compounds, benzoates (Dietz, 1980; Stanier, Doudoroff, and Adelberg, 1970)) and to have a wide range of inducible enzymes (Laskin and Lechevalier, 1974). Rhodopseudomonas capsulata is known to transform nitrosamines (carcinogens) to innocuous compounds (168). Some of the purple nonsulfur organisms can grow microaerobically and anaerobically as phototrophs, yet live as heterotrophs aerobically in the dark, by respiratory metabolism of organic compounds (Pfennig, 1978a; Stanier, Doudoroff, and Adelberg, 1970). Cyanobacteria and Chlorella are tolerant of low concentrations of dissolved oxygen (Kobayashi and Rittmann, 1982). Dunaliella can tolerate a wide salinity range. See Table A.3-2 for a summary of the most favorable conditions for these organisms for removal of anthropogenic compounds (Kobayashi and Rittmann, 1982).

In general, phototrophs do not promote complete degradation, but only transformation. Table A.3-1 lists some of the compounds that can be degraded by certain phototrophs. The contribution of these microbes to complete degradation requires interactions with other organisms. However, the metabolic products they form stimulate growth of heterotrophic organisms. The proper balance between algae and bacteria can result in extensive biodegradation of anthropogenic compounds.

Phototrophic organisms can not only break down organic compounds, but they can also bioaccumulate hydrophobic compounds (Table A.3-7) (Kobayashi and Rittmann, 1982). This offers the greatest potential for exploitation in treatment processes because such organisms can be self-sustaining without the

presence of organic matter in concentrations large enough to serve as carbon and electron donors. Some cyanobacteria and photosynthetic bacteria can also fix nitrogen and, thus, survive in a concentration of dissolved nitrogen that is insufficient to support bacterial growth. On the other hand, when xenobiotics accumulate in the cells of microorganisms they can become concentrated and eventually enter into the food chain (de Klerk and van der linden, 1974).

Biodegradation of organic compounds by specific phototrophs is discussed in Appendix A, Section 3.1.3.

3.1.4 Higher Life Forms and Predation

The role of the soil macrofauna, such as insects, protozoa, earthworms, and slugs, in the decomposition of organic materials is significant, but predominantly indirect (Parr, Sikora, and Burge, 1983). It is minor compared with microorganisms, but it is still essential. Of the total respiration associated with soils amended with organic material, 10 to 20 percent could be from macrofauna. Because only a few of these organisms have the ability to produce their own enzymes for the degradation of substrate, their main degradation feature is mechanical.

The gut of most soil animals contains microorganisms, which produce the necessary enzymes for the degradation of a substrate to the point where the animals can absorb the nutrients. The remainder of the substrate passes into the soil where microorganisms complete the degradation.

Earthworms play a prominent role in the degradation of organic materials in soil. With their movement, the soil is aerated and nutrients are carried to deeper soil profiles where these stimulate microbial growth and decomposition. Among the arthropods, the beetles and termites are most correlated with extensive degradation of organic material. Both animals often have a rich microflora in their guts, and these microorganisms produce the enzymes that degrade cellulosic substrates.

Microbial predators also play a role in the degradation process (Texas Research Institute, Inc., 1982). These organisms graze on bacteria and fungi or feed on detrital matter and associated microflora (JRB Associates, Inc., 1984b; Sinclair and Ghiorse, 1985). Protozoa, nematodes, insects, and other worms affect the decomposition process by controlling bacterial or fungal population size through grazing (Bryant, Woods, Coleman, Fairbanks, McClellan, and Cole, 1982), by harboring in their intestinal tract organisms that might decompose a compound of interest, by comminuting plant materials, or by mixing the soil and contributing to its aeration and homogeneity (JRB and Associates, Inc., 1984b). A cyst-forming amoeba was present at 111.1/g dry weight and constituted 15 percent of the total biovolume of sediments in a groundwater interface zone (Sinclair and Ghiorse, 1985). Many species of hydrocarbon utilizers have been found to be ingested by a large number of ciliate and other cytophagic protozoans (Texas Research Institute, Inc. 1982). These higher organisms may reduce the microbial population from 10^7 to 10^2 bacteria/ml (ZoBell, 1973). Protozoan grazing has been shown to be responsible for most of the acclimation period for the mineralization of organic compounds in some sewage (Wiggins and Alexander, 1986).

3.1.5 Cometabolism

Xenobiotic organic compounds are usually transformed or degraded by microorganisms in either a metabolic sequence that provides energy and nutrients (e.g., carbon (C), nitrogen (N), phosphorus (P)) for growth or maintenance of the organism, or by a biochemically mediated reaction that provides neither energy nor nutrients to the cell (JRB and Associates, Inc., 1984b). The first process usually results in the complete biodegradation of the organic molecules to mineral products (e.g., carbon dioxide, methane, water, ammonia, phosphate) and is called mineralization. The second process usually results in only a minor transformation of the organic molecule and is called cometabolism or cooxidation (Pierce, 1982a; Alexander, 1977; Alexander, 1973). This transformation product is still unusable to the organisms (Hornick, Fisher, and Paolini, 1983).

Two or more substrates are required for cometabolism; one is the nongrowth substrate that is neither essential for, nor sufficient to, support replication of the microorganism (Perry, 1979; Hulbert and Krawiec, 1977), while the other compound(s) does (do) support growth. The nongrowth substrate is only incidentally and incompletely transformed by the microorganism involved, although other microorganisms can often utilize by-products of the cometabolic process (Perry, 1979; de Klerk and van der linden, 1974).

Cometabolism is the result of a nonspecific enzyme with a broad substrate specificity attacking a recalcitrant molecule and metabolizing it (de Klerk and van der Linden, 1974; Horvath and Alexander, 1970). The organism supplying the enzyme gains nothing from the metabolic transformation. The enzyme is made by the microorganism to metabolize some other organic compound for its energy (de Klerk and van der linden, 1974; McKenna, 1977). Oxygenases are often involved in cometabolism because they can be induced by, and can attack, a large set of substrates.

Cometabolism probably occurs frequently in natural soil systems, since many genera of bacteria, fungi, and actinomycetes can participate in the process (Alexander, 1977; Horvath and Alexander, 1970). Cometabolism may be important in the biodegradation of complex organics in hazardous waste-contaminated soils. The mixed chemical environment of petroleum permits a variety of microorganisms to enzymatically attack compounds that would not otherwise be degraded, as they grow on the multitude of potential primary substrates in the oil (Atlas, 1981). Many complex branched and cyclic hydrocarbons are removed as environmental contaminants after oil spills, as a result of cooxidation. Structures with four or more condensed rings have been shown to be attacked by cooxidation or as a result of commensalism, where two or more microorganisms are involved in the degradation and one benefits from it.

Since cometabolism rarely results in the complete oxidation of xenobiotics, it may allow accumulation of transformation products, which may be either more or less toxic than the original substances (Perry, 1979; de Klerk and van der linden, 1974). Incompletely degraded compounds include saturated hydrocarbons, halogenated hydrocarbons, many pesticides, and single-ringed polycyclic aromatic hydrocarbons (Alexander, 1977; Horvath and Alexander, 1970; Horvath, 1972). Cooxidation may be encouraged by adding a more easily degraded compound that is a chemical analog to the hazardous compound that must be decomposed. See Appendix A, Section 5.1.1.2, for examples of compounds that

can be used for analog enrichment and organisms that can be added for cooxidation of specific hydrocarbons.

The metabolism of nongrowth substrates in the absence of a true growth substrate is also referred to as stationary metabolism (Foster, 1962). Aside from traditional fermentation and growth of microorganisms on hydrocarbons, cooxidation and stationary transformation techniques and genetic engineering are the most promising areas of hydrocarbon biotechnology for the future (Hou, 1982). These systems are operating in nature.

3.1.6 Microbial Interactions

Microbial interactions in the soil are very complex and undoubtedly play an important role in the transformation or decomposition of hazardous waste components (JRB and Associates, Inc., 1984b). The growth requirements among the organisms lead to intense competition for the available nutrients. As a result of metabolic specialization (e.g., autotrophic nitrification), some microbes are less dependent upon preformed organic substrates or growth factors. The majority of the organisms, however, have acquired antagonistic abilities that help limit growth of competitors. Examples of these antagonistic agents are antibiotics, acids, bases, and other organic and inorganic compounds (Alexander, 1971; Atlas and Bartha, 1981).

True mutualistic or symbiotic relationships also exist among soil organisms (JRB and Associates, Inc., 1984b). It is common for degradation of a xenobiotic compound to involve sequential metabolism by two or more microorganisms (Beam and Perry, 1974) in a relationship that may benefit only one partner (commensalism) or both (protocooperation or synergism) (Atlas adn Bartha, 1981). In such a commensalistic relationship, microbes cannot oxidize a given hydrocarbon individually, but collectively they are able to do so.

This form of commensalism may be very widespread in nature with natural mixed populations employing each other's metabolic intermediates as growth substrates (Donoghue, Griffin, Norris, and Trudgill, 1976). Many genera of bacteria, fungi, and actinomycetes can participate in the process (Alexander, 1977; Horvath and Alexander, 1970). Appendix A, Section 5.1.1.1.1, provides examples of organisms involved in this form of commensalism.

More than 100 strains of bacteria were unable to use unsubstituted cycloparaffinic hydrocarbons as their sole source of carbon and energy; however, many could partially oxidize the hydrocarbons when a suitable energy source was present (e.g., *n*-alkane) (Beam and Perry, 1973; Beam and Perry, 1974). The resulting cycloalkanones were readily oxidized and used as an energy source by other strains of bacteria. Unsubstituted cycloparaffinic hydrocarbons are readily mineralized in natural soil systems, presumably by a process that includes cometabolism.

3.2 MICROORGANISMS IN GROUNDWATER

Surface soils contain many microorganisms, but their numbers decrease rapidly below the root zone (Bitton and Gerba, 1985). Indigenous microbiological activity may extend to a depth of about 2000 m (assuming a normal temperature increase of $3^{o}C/100$ m). Water pressures to this depth would allow their survival, and many bacteria can withstand the high osmotic pressures of saline groundwater. Carbonates and other inorganic carbon compounds present in most underground materials can serve as alternate carbon sources to organic compounds. Other essential elements, such as phosphorus (P), sulfur (S), and trace elements are also generally present in underground materials.

Members of the deep subsurface microbial community range from aerobic heterotrophic bacteria capable of mineralizing a variety of organic substrates (including glucose, acetate, indole, phenol, 4-methoxybenzoic acid, styrene, toluene, chlorobenzene, and bromodichloromethane) (Fredrickson and Hicks, 1987; Ward, Tomson, Bedient, and Lee, 1986) to denitrifying, sulfate-reducing, and methanogenic anaerobic bacteria (Fredrickson and Hicks, 1987). The presence of carboxylated metabolites in groundwater suggests, however, that biotransformation of jet fuel hydrocarbons stops short of complete mineralization to inorganic carbon, presumably reflecting oxygen limitation (Ehrlich, Schroeder, and Martin, 1985). Fungi, protozoa, sporeforming bacteria, and autotrophic bacteria have also been found. Contrary to the perception that deep sediments are highly reduced and anaerobic, microbiological activity and pore water chemistry actually indicate a relatively oxidized environment. Populations of aerobic heterotrophs are dominant.

Table A.3-8 presents some of the growth requirements for microorganisms in the groundwater environment.

3.2.1 Bacteria

Bacteria have been found at significant levels in groundwater samples and have been the predominant species (Larson and Ventullo, 1983). The origin of bacteria in aquifers is unknown (Bitton and Gerba, 1985). They may have been deposited with the sediments millions of years ago, migrated recently into the formations with surface water or through soil fissures, or been introduced during construction of wells. Many microbes seem to move passively through soil pores larger than their cell size.

Since groundwater from deep, protected aquifers is considered oligotrophic, the bacteria in this region must be capable of metabolism in low nutrient concentrations (Stetzenbach, Kelley, and Sinclair, 1984). Water collected from wells 200 ft deep contained predominantly gram-negative rods, with a limited number of genera. These were mostly Acinetobacter. Both gram-negative and gram-positive bacteria were recovered from depths of 1.2, 3.0, and 5.0 meters, where the water table was at 3.6 meters and the bedrock at 6.0 meters (Wilson, McNabb, Balkwill, and Ghiorse, 1983). Indigenous bacterial populations present in groundwater have the potential to metabolize both natural and xenobiotic substances, depending upon the suitability of the substrate as a carbon and energy source (Larson and Ventullo, 1983). For degradable substrates, rates of biodegradation are fairly rapid, even in oligotrophic groundwater.

3.2.1.1 Aerobes

Of 500 isolates recovered from groundwater from deep, protected aquifers, more than 80 percent were gram-negative, nonmotile, rod-shaped bacteria (Stetzenbach, Sinclair, and Kelley, 1983). Some 59 percent of the isolates were Acinetobacter spp. Species of Moraxella, Flavobacterium, and an unidentified oxidase-negative, pigmented bacterium were also present. Corynebacterium species may be major agents for decomposing heterocyclic compounds and hydrocarbons in contaminated aquatic environments (Kobayashi and Rittmann, 1982).

Oxygen levels in groundwater samples contaminated with gasoline and fuel oil ranged from trace amounts to 4 ppm; however, cultures grown under microaerophilic conditions resembled aerobic forms (Litchfield and Clark, 1972). The numbers of hydrocarbon-utilizing bacteria correlated positively with the levels of contamination, suggesting these organisms were growing in the groundwater. Major isolates from a variety of contaminated groundwaters were species of Pseudomonas, Arthrobacter, and Nocardia, and occasionally Achromobacter and Flavobacterium.

3.2.1.2 Anaerobes

As much as 2 to 8 mg/l have been found in water from deep aquifers in Arizona and Nevada (Winograd and Robertson, 1982). Sometimes deep aquifers (several hundred feet) contain high levels of oxygen because of the absence of organics. Except in very deep, isolated formations, oxidation-reduction potentials tend to be within the tolerance range of aerobic bacteria (Bitton and Gerba, 1985). When molecular oxygen is absent in deeper regions, anaerobic bacteria can be found. These include sulfate reducers, denitrifiers, methanogens, and sulfur and hydrocarbon oxidizers (McNabb and Dunlap, 1975). These bacteria use sulfate, carbon dioxide, nitrate, and simple organic compounds rather than oxygen as electron acceptors in their metabolism.

If oxygen, nutrients, or carbon source of energy is depleted in a contaminated aquifer, anaerobic bacteria can become active in biotransformations (Wilson, Leach, Henson, and Jones, 1986). Anaerobic bacteria are generally unable to utilize most of the compounds in crude oil and its refined derivatives as sources of carbon and nutrients (Ehrlich, Schroeder, and Martin, 1985). However, some biotransformation of hydrocarbons appears to be occurring (e.g., conversion of alkanes to fatty acids), even though there is not a complete oxidation of most hydrocarbons to carbon dioxide and water (Widdle and Nobert, 1981).

Methane-producing bacteria can assimilate only a limited range of compounds; they are capable of autotrophic growth with hydrogen and carbon dioxide, and some species can use formate, acetate, or methanol as growth substrates (Ehrlich, Schroeder, and Martin, 1985). Sulfate-reducing bacteria can utilize certain short-chain fatty acids, including acetate (Widdle and Nobert, 1981). Molecular hydrogen can also be used to reduce inorganic carbonate to methane (Wilson, Leach, Henson, and Jones, 1986). The presence of fatty acids and benzoate in water from some wells suggests that an incomplete biotransformation of hydrocarbons is occurring in the shallow zone. This may reflect an oxygen limitation (Widdle and Nobert, 1981).

Methane-producing bacteria and dissolved methane were detected in water samples from two wells from a JP-5-contaminated aquifer, in spite of exposure to oxygen during well recovery following bailing (Ehrlich, Schroeder, and Martin, 1985). Methane-producing bacteria may be less sensitive to oxygen toxicity than is commonly supposed.

It has recently been found that compounds, such as benzene, toluene, the xylenes, and other alkylbenzenes, do not require molecular oxygen to be metabolized (Wilson, Leach, Henson, and Jones, 1986). They could be at least partially metabolized in methanogenic river alluvium to carbon dioxide. Phenolics can also be degraded anaerobically (Rees and King, 1980); however, aromatic compounds are not significantly utilized under anaerobic conditions (Bouwer and McCarty, 1983b). Although strict anaerobes that produce methane can use a very limited range of organic compounds, they can act in combination with other microorganisms to break down more complex organic compounds (Wilson, Leach, Henson, and Jones, 1986). These partnerships can totally degrade a surprising variety of natural and synthetic organic compounds.

The different types of anaerobic degradation are explained in Appendix A, Section 4.1.2.

3.2.1.3 Counts in Uncontaminated Groundwater

Special staining procedures can distinguish cellular material from noncellular subsurface soil particles of the same size and shape (Ghiorse and Balkwill, 1983). This technique was used to count microbes present in core material from shallow water table aquifers and vadose zones. Bacteria, but no yeasts or other fungi, protozoa, or higher animals were found. Tables A.3-9 and A.3-10 show the similarity of counts recovered from different depths.

Appreciable numbers of bacteria (up to 10^8 cells/g of dry soil) have been found to exist in shallow, uncontaminated water table aquifers (Wilson, McNabb, Balkwill, and Ghiorse, 1983; Fredrickson and Hicks, 1987; McNabb and Dunlap, 1975; White, Smith, Gehron, Parker, Findlay, Matz, and Fredrickson, 1982), as well as in deep aquifers (White, Smith, Gehron, Parker, Findlay, Matz, and Fredrickson, 1982). Viable bacteria that degrade naturally occurring compounds range from 0.01 to 10^6/g of soil (Swindoll, Aelion, Dobbins, Jiang, Long, and Pfaender, 1988) (see Appendix A). The xenobiotic degraders were generally less than 10/g of soil.

3.2.1.4 Counts in Contaminated Groundwater

Numbers of hydrocarbon utilizers were found to increase in sites after contamination. After a gasoline spill in Southern California in 1972, samples from wells that had traces of free gasoline yielded 5 x 10^4 gasoline-utilizing bacteria/ml or higher; while an uncontaminated well had only 200 organisms/ml (McKee, Laverty, and Hertel, 1972). A hazardous waste site contaminated with high concentrations of jet fuel hydrocarbons, industrial solvents, and heavy metals had bacterial counts of 10^7 cells/wet g sample from both the unsaturated and the saturated zones.

Groundwater samples collected throughout the United States from aquifers contaminated with hydrocarbons were analyzed and found to contain hydrocarbon-utilizing bacteria at levels of up to 10^6 organisms/ml (Litchfield and Clark,

1973). Significant populations of these organisms are present in groundwaters contaminated with gasoline, fuel oil, and other mixed petroleum products. Groundwaters containing less than 10 ppm of gasoline, fuel oil, and other petroleum products generally had populations of less than 10^3 bacteria/ml (Environmental Protection Agency, 1985b). Concentrations of these hydrocarbons in excess of 10 ppm sometimes supported growth of 10^6 bacteria/ml. Acridine orange direct counts, standard methods agar, and JP-5 agar plate counts were higher for samples from contaminated (10^6 to 10^7 cells/ml) than from uncontaminated (2×10^4 cells/ml) wells (Ehrlich, Schroeder, and Martin, 1985). They were highest in contaminated wells with a free fuel layer.

3.3 MICROORGANISMS IN LAKE, ESTUARINE, AND MARINE ENVIRONMENTS

More than 10 million metric tons of petroleum pollutants are estimated to contaminate the world's oceans each year (Atlas, 1978c). Most of these pollutants come from routine operations, such as release of oil from tanker ballast. Relatively little is contributed by catastrophic accidents. Petroleum biodegradation is also a naturally occurring process, which may account for the fact that not all of the world's surface waters are completely covered with a layer of oil (Gholson, Guire, and Friede, 1972). Evaporation and biodegradation of petroleum hydrocarbons are the major processes that remove petroleum pollutants from the contaminated ecosystem (Atlas, 1978c). The biodegradation of the complex mixture of hydrocarbons in petroleum is generally incomplete, leaving a highly asphaltic residue that can form very persistent tar balls.

Microbes are important in degrading petroleum hydrocarbons in ocean waters (Lee and Ryan, 1976). The addition of hydrocarbons to an ecosystem, as occurs from oil spillages, may result in selective increases or decreases in the microbial populations (Bartha and Atlas, 1977). Addition of different oil fractions to seawater results in increases in the populations of different bacterial species (Horowitz, Gutnick, and Rosenberg, 1975). Petroleum hydrocarbons also select for particular species of hydrocarbonoclastic microorganisms (Zajic and Daugulis, 1975). Both oil type and temperature are important in causing differential population increases (Westlake, Robson, Phillippe, and Cook, 1974).

Bacteria and yeasts appear to be the prevalent hydrocarbon degraders in aquatic ecosystems (Bartha and Atlas, 1977). The most important genera (based upon frequency of isolation) of hydrocarbon utilizers in aquatic environments are *Pseudomonas*, *Achromobacter*, *Arthrobacter*, *Micrococcus*, *Nocardia*, *Vibrio*, *Acinetobacter*, *Brevibacterium*, *Corynebacterium*, *Flavobacterium*, *Candida*, *Rhodotorula*, and *Sporobolomyces*. *Corynebacterium* species may be major agents for decomposing heterocyclic compounds and hydrocarbons in contaminated aquatic environments (Kobayashi and Rittmann, 1982).

In agitated enrichments, bacteria tend to take over, but some investigators believe that fungal forms can be significant or even predominant in undisturbed surface slicks (Bartha and Atlas, 1977). In polluted freshwater ecosystems, bacteria, yeasts, and filamentous fungi all appear to be important hydrocarbon degraders (Cooney and Summers, 1976). Table A.3-12 lists the predominant hydrocarbon-degrading microorganisms found in aquatic environments.

Marine and estuarine bacteria appear to be susceptible to lysis with fluctuations in salinity and osmotic pressure (Ahearn, Meyers, and Standard, 1971). Increased numbers and localization of strongly hydrocarbonoclastic yeasts in oil-bearing regions suggest their *in situ* activity in oil biodegradation processes. Vegetative yeast cells are more resistant than those of bacteria to variable salinity, osmotic pressure, and ultraviolet rays. Oil-degrading yeast strains isolated from freshwater are also active in seawater.

Field and laboratory data suggest that yeasts play a role in microbial decomposition of surface oil depositions in the marine environment (Ahearn, Meyers, and Standard, 1971). However, their activity is greatly affected by the concentration of hydrocarbons. Yeasts of the genera *Candida*, *Trichosporon*,

Rhodosporidium, *Rhodotorula*, *Debaryomyces*, *Endomycopsis*, and *Pichia*, isolated from all marine environments, readily assimilated hexadecane and kerosene at a concentration of 2 percent; however, many of the strains did not grow well on 4 percent hydrocarbon. Such strains are generally concentrated in oil-polluted habitats.

Aquatic algae have also been implicated in degradation of organic chemicals. Algae may degrade organic compounds by mediation of accelerated photolysis or by uptake and degradation of the molecule with intracellular enzymes. Section 3.1.3 discusses these processes. A greater number of heterotrophic microorganisms can be supported in dense algal communities. The achlorophyllous algae *Prototheca hydrocarbonea* and *P. zopfii* can degrade hydrocarbons without light, while *Scenedesmus* strains can utilize *n*-heptadecane in the light only (Masters and Zajic, 1971).

The spillage of oil in the marine environment is a severe stress to that ecosystem because of the sudden large increase in organic matter (Atlas, 1977). Marine microorganisms are generally adapted to low levels of organic matter. Appendix A, Section 3.3 explains what can happen to a microbial community with the sudden appearance of high concentrations of organics as a result of an oil spill.

Concentrations of microorganisms in marine environments are relatively low compared with other ecosystems. Therefore, seed microorganisms would be able to compete with the naturally occurring microorganisms in an ecosystem very different from the one to which the indigenous microorganisms were adapted.

Temperature and chemical composition of contaminants have a selective influence on the genera of hydrocarbon utilizers (Atlas, 1981). Different combinations of *Achromobacter*, *Alcaligenes*, *Flavobacterium*, *Cytophaga*, *Acinetobacter*, *Pseudomonas*, *Arthrobacter*, and *Xanthomonas* were isolated from crude oil samples, depending upon the source of the sample and temperature of incubation (4^{o} or 30^{o}C) (Cook and Westlake, 1974). Several gram-negative and gram-positive thermophilic hydrocarbon-utilizing bacteria have also been recovered, including species of *Thermomicrobium* (Merkel, Stapleton, and Perry, 1978). Some thermophiles are obligate hydrocarbon utilizers and cannot grow on other carbon sources.

Psychrophilic isolates that utilized paraffinic, aromatic, and asphaltic petroleum components were found in an asphaltic flow near a natural seepage in Alaska (Jensen, 1975). They belong to the genera *Pseudomonas*, *Brevibacterium*, *Spirillum*, *Xanthomonas*, *Alcaligenes*, and *Arthrobacter*. Hydrocarbon-utilizing *Nocardia*, *Flavobacterium*, *Vibrio*, *Pseudomonas*, and *Achromobacter* were present in northwest Atlantic coastal waters and sediment (Walker, Colwell, and Petrakis, 1976).

Chronic exposure to very high concentrations of degradable hydrocarbons can greatly increase the activity and numbers of hydrocarbon-degrading microorganisms (Heitkamp and Cerniglia, 1987). Water samples from oil-contaminated sections of lakes were better able to degrade test hydrocarbons than water from noncontaminated parts of the same lakes, presumably as a result of enrichment of hydrocarbon-using microorganisms in the former (Boyle, Finger, Petty, Smith, and Huckings, 1984). An aquatic environment exposed to petroleum hydrocarbons had a larger population of hydrocarbon-degrading microorganisms than an uncon-

taminated environment, even though the latter had higher numbers of heterotrophic microorganisms (Heitkamp and Cerniglia, 1987). Not all heterotrophs are able to use organic compounds as substrates and plate counts (or other enumerative procedures that require growth on artificial media) of total heterotrophic microbial populations may not be a reliable indicator of the hydrocarbon-degrading potential of aquatic sediments. PAHs with less than four aromatic rings can be degraded in freshwater and estaurine ecosystems, but higher molecular weight PAHs, such as benzo(a)pyrene, persist for relatively long periods of time, even in ecosystems microbially adapted to aromatic hydrocarbons.

3.3.1 Counts

The number of oil degraders in a natural body of water is chiefly determined by the pollution history (Bartha and Atlas, 1977). See Appendix A, Section 3.3.1, for the bacterial counts found in aquatic environments.

SECTION 4

MICROBIAL DEGRADATION AND TRANSFORMATION OF PETROLEUM CONSTITUENTS AND RELATED ELEMENTS

There are three processes by which microorganisms can break down hydrocarbons: fermentation, aerobic respiration, and anaerobic respiration (Canter and Knox, 1985). In fermentation, the carbon and energy source is broken down by a series of enzyme-mediated reactions that do not involve an electron transport chain. In aerobic respiration, the carbon and energy source is broken down by a series of enzyme-mediated reactions in which oxygen serves as an external electron acceptor. In anaerobic respiration, the carbon and energy source is broken down by a series of enzyme-mediated reactions in which nitrates, sulfates, carbon dioxide, and other oxidized compounds (excluding oxygen), serve as external electron acceptors. These three processes of obtaining energy form the basis for the various biological wastewater treatment processes.

Table A.4-2 summarizes the different ways these processes are used by the organisms and shows the relationship between the processes and redox potential. Appendix A, Section 4, also elaborates on the differences between heterotrophic and autotrophic metabolism.

4.1 ORGANIC COMPOUNDS

The ability of certain microorganisms to oxidize simple aromatic hydrocarbons has been demonstrated (Table A.4-3) (Gibson, 1977). Most of our present knowledge of the microbial degradation of aromatic hydrocarbons has been obtained with single hydrocarbon substrates and pure cultures of different microorganisms (Tables A.4-4 and A.4-5). Little is known about how microorganisms interact with compounds when they are present in a sample of petroleum.

At a given density of oil-degrading microorganisms, their actual contribution to the elimination of oil depends upon their inherent metabolic capability; i.e., "heterotrophic potential," and the degree to which environmental conditions allow this potential to be expressed (Bartha and Atlas, 1977).

Petroleum is chemically very complex, consisting of hundreds of individual organic compounds (Atlas, 1977). For microorganisms to completely biodegrade petroleum or attack even simpler refined oils, thousands of different compounds may be involved, which must be metabolized. The chemical nature of these petroleum components varies from the simple n-paraffin, monoalicyclic, and monoaromatic compounds, to the much more complex branched chains and condensed ring structures (Horowitz, Gutnick, and Rosenberg, 1975). Many different enzymes are necessary to biodegrade these types of compounds.

The principal biochemical reactions associated with the microbial metabolism of xenobiotics include acylation, alkylation, dealkylation, dehalogenation, amide or ester hydrolysis, oxidation, reduction, hydroxylation, aromatic ring cleavage, and condensation and conjugate formation (Kaufman, 1983).

4.1.1 Aerobic Degradation

Aerobic degradation in soil is dominated by a variety of organisms, including bacteria, actinomycetes, and fungi, which require oxygen during chemical degradation (Parr, Sikora, and Burge, 1983). This process involves oxidation-reduction reactions in which molecular oxygen serves as the ultimate electron acceptor, while an organic component of the contaminating substance functions as the electron donor or energy source in heterotrophic metabolism. Appendix A, Section 4.1.1, explains the differences between aerobic oxidation of aromatic hydrocarbons by eukaryotic and procaryotic organisms (Gibson, 1977). Most aerobic bacteria use oxygen to decompose organic compounds into carbon dioxide and other inorganic compounds (Freeze and Cherry, 1979). In soil, oxygen is supplied through diffusion. If the oxygen demand is greater than the rate of oxygen diffused, the soil naturally becomes anaerobic. Maximum degradation rates for petroleum hydrocarbons are dependent upon the availability of molecular oxygen. Aerobic biodegradation occurs *via* more efficient and rapid metabolic pathways than anaerobic reactions (Zitrides, 1983). Therefore, most site decontaminations involving refined oils and fuels are conducted under aerobic conditions.

Metabolic pathways have been established for the degradation of a number of simple aliphatic and aromatic structures (Atlas, 1978c). The general degradation pathway for an alkane involves sequential formation of an alcohol, an aldehyde, and a fatty acid. The fatty acid is then cleaved, releasing carbon

dioxide and forming a new fatty acid two carbon units shorter than the parent molecule. This process is known as beta oxidation. The initial enzymatic attack involves a class of enzymes called oxygenases. The general pathway for degradation of an aromatic hydrocarbon involves cis-hydroxylation of the ring structure forming a diol, e.g., catechol. (In the cis configuration, the hydroxyl groups are on the same side of the molecule.) The ring is then oxidatively cleaved by oxygenases, forming a dicarboxylic acid, e.g., muconic acid.

Degradation of substituted aromatic compounds generally proceeds by initial beta oxidation of the sidechain, followed by cleavage of the ring structure. Simple alkyl substitution of benzene generally increases its rate of degradation, but extensive alkylation inhibits degradation. Branching generally retards the rate of alkane degradation. It may also change the metabolic pathway for utilization of a hydrocarbon (Pirnik, 1977). Some long chain alkanes may be degraded by different metabolic pathways, such as subterminal oxidation (Markovetz, 1971). The degradative pathway for a highly branched compound, such as pristane or phytane, may proceed by omega oxidation, forming a dicarboxylic acid instead of only monocarboxylic acids, as in normal beta-oxidation. Appendix A, Section 4.1.1, discusses the reactions involved in these processes and describes some of the major pathways microorganisms use for aerobic degradation.

Appendix A presents individual hydrocarbons and some of the organisms that can degrade them (Section 4.1.1) and some end products of the reactions (Section 4.1.3).

4.1.2 Anaerobic Degradation

Anaerobic microbial transformations of organic compounds are important in anoxic environments (Young, 1984; Sleat and Robinson, 1984). Less cell material is formed under anaerobic conditions because of the lower growth yield, but organic fermentation products are likely to accumulate, unless they are converted into methane, or other hydrocarbon gases. Anaerobiosis usually occurs in any habitat in which the oxygen consumption rate exceeds its supply rate and is a common phenomenon in many natural aquatic environments receiving organic materials (Berry, Francis, and Bollag, 1987). Examples include flooded soils and sediments, eutrophic lagoons, stagnant fresh and ocean waters, and some groundwaters.

Petroleum can be microbially degraded anaerobically by the reduction of sulfates and nitrates (Shelton and Hunter, 1975). Aromatic hydrocarbons, common to many fuels, that can be biodegraded without the presence of molecular oxygen, include toluene, xylene, alkylbenzenes, and possibly benzene (Grbic-Galic and Young, 1985; Reinhard, Goodman, and Barker, 1984; Kuhn, Colberg, Schnoor, Wanner, Zehnder, and Schwarzenback, 1985).

Some xenobiotic compounds can be degraded under anaerobic conditions *via* fermentation, anaerobic respiration, and photometabolism, both by single species of microbes and by microbial communities (Harder, 1981). Single species have been shown to degrade aromatic compounds in the presence of nitrate or light (Evans, 1977), whereas, a number of microbial communities have been described that degrade these compounds in association with methane production (Balba and Evans, 1977) or in the presence of nitrate (Bakker,

1977). Interactions between different microbial populations are potentially important in the degradation of complex molecules (Hornick, Fisher, and Paolini, 1983).

4.1.2.1 Anaerobic Respiration

Depending upon which of the electron acceptors (e.g., NO_3^-, SO_4^{-2}, or CO_2) is dominant in an anoxic environment, anaerobic respiration may be employed to degrade organic compounds (Berry, Francis, and Bollag, 1987). This may be performed by denitrifying bacteria, sulfate reducing bacteria, or methanogens. The processes by which these organisms degrade hydrocarbons are described in Appendix A, Section 4.1.2.1.

4.1.2.2 Fermentation

Many microorganisms that inhabit anoxic environments obtain their energy for growth through fermentation of organic carbon (Schnitzer, 1982). Fermentation is a process that can be carried out in the absence of light by facultative or obligatory anaerobes. In fermentation, organic compounds serve as both electron donors and acceptors. The process is further described in Appendix A, 4.1.2.2.

4.1.2.3 Specific Compounds

Appendix A, Section 4.1.2.3, presents specific examples of hydrocarbons and the organisms that can degrade them anaerobically, intermediate and end products of the process, and various factors affecting anaerobic biodegradation.

4.1.3 End Products

In soil, organic chemicals are subject to alteration by biochemical reactions that are catalyzed by enzymes from a wide range of organisms (Kaufman, 1983). In general, metabolites arising from these microbial reactions are usually nontoxic, polar molecules that exhibit little ability to accumulate in food chains. However, the breakdown products of many chemicals can be toxic; sometimes they are even more toxic than the parent compound.

In addition to carbon dioxide and water, the products resulting from complete mineralization of hydrocarbons, there are various hydroperoxides, alcohols, phenols, carbonyls, aldehydes, ketones, and esters that result from incomplete oxidation (ZoBell, 1973). The biodegradation of aromatic hydrocarbons yields phenolics and benzoic acid intermediates (Bartha and Atlas, 1977). Complete oxidation is more likely when a diverse mixture of microbes is available (Texas Research Institute, Inc., 1982). The tendency for primary and intermediate oxidation products to accumulate is much greater for a pure culture than a mixed culture. The biodegradation of higher molecular weight hydrocarbons involves many intermediates, some of which may accumulate to inhibitory levels (Bartha and Atlas, 1977). It was found that C_5 to C_9 alkanes were not toxic to a population of bacteria, but that the alcohols of these hydrocarbons were inhibitory.

As oxygen in the soil is depleted, microbial reactions become anaerobic with the production of malodorous compounds, such as amines, mercaptans, and

H_2S, which can be phytotoxic. However, under aerobic conditions, the end products will be inorganic carbon, nitrogen, phosphorus, and sulfur compounds.

Table A.4-7 lists the products formed from the action of various microorganisms on specific hydrocarbons.

4.2 HEAVY METALS AND METALLOIDS

The toxic nature of arsenic (As), barium (Ba), cadmium (Cd), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni), selenium (Se), silver (Ag), thallium (Tl), and vanadium (V) can adversely affect microbial populations when the soil is contaminated (JRB and Associates, Inc., 1984b). These inorganic elements cannot be destroyed; however, they can be recovered and recycled (Scholze, Wu, Smith, Bandy, and Basilico, 1986). Ag and Hg are the most toxic to microorganisms, followed by Cd, Zn, Cu, Cr, Pb, and Ni at higher concentrations (Josephson, 1983). Some chemicals will be more toxic and biocidal to the soil microflora than others, and they may cause major changes in microbial populations that could persist for weeks or indefinitely. The effect of the metals in waste on the soil microorganisms present should be determined. Industrial wastes are a major source of metals that could be inhibitory to the degradation of petroleum products, such as by inhibition of enzyme activity. This toxicity can be alleviated by limestone application, if the metal loadings are restricted. However, the effects of heavy loadings of metals are difficult to correct, and the soil may have to be removed.

Some microorganisms show a tolerance for heavy metals (Sims and Bass, 1984; Monroe, 1985). They may require low levels of heavy metals for their microbial enzyme systems (Parr, Sikora, and Burge, 1983) and may use redox-sensitive metals and metalloids (e.g., iron, manganese, selenium) as a source of energy and respiration (Monroe, 1985). They may be able to oxidize, reduce, methylate, demethylate, or otherwise transform these elements so their solubility, sorption, or volatility in the soil is greatly affected (JRB and Associates, Inc., 1984b). Microorganisms that play an important role in oil degradation have often been able to utilize some heavy metals (e.g., iron and manganese), even at high concentrations, as energy sources or electron acceptors in their respiratory processes (Hornick, Fisher, and Paolini, 1983). These reactions may involve precipitation, adsorption, or volatilization of the metals, thereby, making the environment more favorable for other microbial species (Ehrich, 1978).

Biodegradation is generally used as a detoxification mechanism for organic contaminants in soil; however, it may also have applications in treating contaminations where heavy metals are present (Sims and Bass, 1984). Some organisms are even employed for extraction and recovery of different metals (Monroe, 1985). The bacteria, fungi, or algae utilized and the metals recovered are shown in Table A.4-8.

Appendix A, Section 4.2, describes the mechanisms used by microorganisms for resistance to metals and the measures that can be taken to increase metal biotransformation. It lists the elements present in the fuels addressed in this review and discusses the potential for using microbes in contamination incidents.

SECTION 5

ENHANCEMENT OF BIODEGRADATION

5.1 OPTIMIZATION OF SOIL BIODEGRADATION

Some of the information in this section may duplicate material covered in Section 5.2, Optimization of Groundwater Biodegradation, and Section 5.3, Optimization of Freshwater, Estaurine, and Marine Biodegradation; however, it is presented here under a separate heading, with other related information, to accommodate those readers who may specifically wish to address treatment of soil contamination only.

An in-depth evaluation of the potential for microorganisms to remove anthropogenic organic compounds from a contaminated environment indicates that use of properly selected populations of microbes, and the maintenance of environmental conditions most conducive to their metabolism, can be an important means of optimizing biological treatment of organic wastes (Kobayashi and Rittmann, 1982).

Most bacteria in the subsurface are firmly attached to soil particles (Wilson, Leach, Henson, and Jones, 1986). As a result, nutrients must be brought to the microbes by advection or diffusion through the mobile phases, i.e., water and soil gas. In the simplest and perhaps most common case, the organic compound to be consumed for energy and cell synthesis is brought into aqueous solution by infiltrating water. At the same time, oxygen, the electron acceptor used to oxidize the carbon source is brought by diffusion through the soil gas. In the unsaturated zone, volatile organic compounds can also move readily as vapors in the soil gas. Below the water table all transport must be through liquid phases, and as a result, the prospects for aerobic metabolism are severely limited by the very low solubility of oxygen in water. In the final analysis, the rate of biological activity is controlled by:

1. The stoichiometry of the metabolic process

2. The concentration of the required nutrients in the mobile phases

3. The advective flow of the mobile phases or the steepness of concentration gradients within the phases

4. Opportunity for colonization in the subsurface by metabolically capable organisms

5. Toxicity exhibited by the waste or a co-occurring material.

Biological restoration of contaminated soils and aquifers has been accomplished using several techniques. Suntech pioneered the use of "bioreclamation" to restore petroleum-contaminated aquifers (Raymond, 1974; Raymond, 1978; Raymond, Jamison, and Hudson, 1976). This process enhances the activity of the natural microbial population in soil and groundwater by providing dissolved oxygen and nutrients to accelerate biodegradation. This technology of enhanced biodegradation has now been significantly modified for use at

hazardous waste sites (JRB and Associates, Inc., 1982; Jhaveria and Mazzacca, 1982).

It might be possible to enhance the degradation of petroleum if the microbial population can be altered by seeding with mutant or acclimated organisms in a mixture that collectively can degrade all the components of the contaminant (JRB Associates, Inc., 1982; Jhaveria and Mazzacca, 1982). The indigenous hydrocarbon-utilizing microflora of a contaminated site has been enhanced by the recirculation of pumped groundwater containing nutrients and oxygen. The groundwater is injected into wells and circulated through the contaminated zone by pumping groundwater from one or more producing wells (Wilson, Leach, Henson, and Jones, 1986). The environment can be modified to better support the natural microbial activity by altering soil factors; or the petroleum can be modified to make it more susceptible to biodegradation. The surface area available for biodegradation may be increased by dispersing the contaminant, or by emulsification (Atlas, 1977). The "ideal" conditions for efficient utilization of hydrocarbons by microbes includes the following (Texas Research Institute, 1982):

1. A large number and variety of hydrocarbon-utilizing microbes
2. A low population of microbial predators
3. Sufficient oxygen (>2 to 3 ppm in water phase)
4. A high degree of dispersal of the hydrocarbon in the aqueous medium
5. A temperature between 20 and 35^{o}C
6. Sources of nitrogen, phosphorus, and trace minerals
7. A source of readily assimilated organic material
8. A means of removing waste products
9. A nontoxic concentration of hydrocarbons in the aqueous medium
10. A pH of 5 to 9.

In order to improve biological treatment by the use of selected organisms, major issues, such as which organism to select, development of the population, and retention of organisms, must be faced (Kobayashi and Rittmann, 1982). Genetic engineering can be used to develop specialized organisms, but control of environmental factors offers a more practical means of encouraging only the desired species. Sometimes it is necessary to selectively maintain a series, or consortium, of microorganisms to achieve complete degradation (Pfennig, 1978a).

Fixed-film processes (bioreactors) may be the best means to treat wastes above ground to assure population retention and avoid total loss of slow-growing microorganisms (Kobayashi and Rittmann, 1982). The cell concentrations in these fixed-film bioreactors are higher than those found in suspended growth systems. Efficient removal of the organic compounds is possible only when the biomass concentration is large (Matter-Muller, Gujer, Giger, and Strumm, 1980).

An important goal is to establish which organisms carry out the desired reactions and assure their proliferation.

Another factor to consider before artificially stimulating oil biodegradation in soil is whether the processes could allow growth of microorganisms that might adversely affect nearby plants and crops as well as livestock and other animals (Atlas, 1977). Ultimately, how much fertilizer to add and its precise chemical formulation; how much forced aeration to use; and the extent to which other factors have to be modified are questions that must be answered by measuring levels of success following actual testing of various treatments.

5.1.1 Biological Enhancement

Controlling environmental factors to encourage only the desired species is a practical method for assuring proliferation and maintenance of selected populations. The factors that must be manipulated vary with the physiological needs of the organisms. It must be determined which microorganism carries out the limiting reaction and assure its proliferation, at the same time considering interactions with other organisms. In some cases, a series of biological reactors--each maintained under different environmental conditions--is required to provide the wide variety of microorganisms necessary for complete biodegradation.

Since the organisms may grow slowly, population retention is important (Kobayashi and Rittmann, 1982). They could be washed out (total loss of the organism from a reactor) or displace through competition from other organisms. Fixed-film processes may be the best mechanism to assure population retention, when appropriate.

5.1.1.1 Seeding of Microorganisms

Biological treatment methods for petroleum hydrocarbon remediation generally rely upon the stimulation and natural selection of indigenous microorganisms in the soil or groundwater (Sims and Bass, 1984). However, the natural soil flora may not have the metabolic capability to readily degrade certain compounds or classes of compounds or to emulsify the water-insoluble components. On the other hand, they may have the ability but not the biomass necessary to degrade the compounds rapidly enough to meet treatment criteria. In such cases, it might be advisable to add large numbers of exogenously grown microorganisms to the soil.

Considering the diversity of enzymatic activities required, it has been assumed by some that the natural microbial communities of many ecosystems would not possess all the needed enzymes, and, therefore, contaminating oil would not be extensively biodegraded unless hydrocarbon-degrading microorganisms were artificially added (Gholson, Guire, and Friede, 1972). In addition, many of the components would probably be recalcitrant to microbial attack.

The addition of cultures of organisms could reduce the lag period required for indigenous populations to respond to petroleum pollutants (Atlas, 1978c). Some workers have suggested that the natural lag or response time is generally short, in which case, seeding would not usually reduce the impact of petroleum pollutants, except in a few open environments with limited microbial populations.

A special culture collection was begun as a depository for "hydrocarbonoclastic" (hydrocarbon-utilizing) microorganisms (Cobet, 1974). "Superbugs" are being sought to solve the world's pollution problems (Gwynne and Bishop, 1975). Several commercial enterprises are marketing microorganism preparations for removing petroleum pollutants (Atlas and Bartha, 1973e; Azarowicz, 1973). A hydrocarbonoclastic superbug must be able to extensively degrade most of the components found in petroleum. It must be genetically stable, capable of being stored for long periods of time, and able to reproduce rapidly following storage. It must be capable of enzymatic activity and growth in the environment in which it is to be used, and it must be capable of competing with the naturally occurring microorganisms in that environment for a period sufficient to clean up the site. Finally, it must not produce adverse side effects; it must not be pathogenic or produce toxic metabolic end products.

Creation of a single superbug that combines the genetic information from many microorganisms into one could overcome the problem of interference of organisms with the metabolic activities of each other (Friello, Mylroie, and Chakrabarty, 1976). It will be very difficult to engineer a single organism with all these characteristics or even one capable of metabolizing most petroleum components (Atlas and Bartha, 1973e; Azarowicz, 1973). Therefore, it has been proposed that a mixture of microorganisms should be used as the seed culture for oil biodegradation (Kator, 1973). Each organism would be included for its ability to degrade particular petroleum components and would still have to meet the rest of the above criteria.

Microbes can be added to in situ treatment processes to enhance biodegradation by increasing biomass or by reducing the time necessary for acclimation to occur (Lee and Ward, 1986). Usually natural soil microorganisms that have been previously acclimated to degrade the contaminants are used as "seed"; the microbes may have been selected by enrichment culturing, induced mutation, or genetic manipulation (Ward and Lee, 1984). Organisms selected by enrichment culturing have their tolerance and metabolic activity to a particular substance built up over time (Lee and Ward, 1986). The types of microorganisms that are isolated depend upon the source of the inoculum, the conditions used for the enrichment, and the substrate (Atlas, 1977). Microorganisms can become acclimated to specific compounds by repeated exposure to the petroleum product, to certain components, or to related compounds (Zajic and Daugulis, 1975).

Sequential enrichment techniques (see Appendix D) are a modification of enrichment culturing and can be used to isolate microorganisms capable of degrading most of the components of petroleum (Environmental Protection Agency, 1985b; Horowitz, Gutnick, and Rosenberg, 1975). The substrate is inoculated with a microbial population, and the organisms that can degrade it are isolated. The undegraded substrate is then used to isolate another set of organisms that are capable of degrading the residual components. This continues until none of the substrate remains or no new isolates are recovered. Different mixtures of organisms may be isolated from soils, if the enrichments are carried out at 4^{o} rather than $20^{o}C$ (Jobson, Cook, and Westlake, 1972).

The resulting mixture of organisms is more effective than the individual isolates. However, organisms isolated individually in the sequential enrichments may not be able to degrade the oil simultaneously; one organism in

the mixture may interfere with another. This process may allow isolation of various microorganisms that could degrade the low solubility, high molecular weight, as well as the more soluble and toxic, hydrocarbons and intermediates of hydrocarbon metabolism (Zajic and Daugulis, 1975). Such selective continuous enrichments may be occurring in nature in areas subjected to constant input of petroleum hydrocarbons. Since intermediary metabolites must also be removed for complete oil cleanup, nonhydrocarbon-utilizing microorganisms, such as fatty acid metabolizers, would also be required in the mixture (Atlas, 1977).

Seeding microorganisms onto soil was first tried around 1968 (Gutnick and Rosenberg, 1979). An inoculum of Cellumonas sp. and nutrients was able to degrade hydrocarbon contaminants more effectively than just fertilizer alone (Schwendinger, 1968). Since then, the seeding of microorganisms has been used in a number of different environments to degrade organics (Environmental Protection Agency, 1985b). Varying success has been obtained when seeding microorganisms onto petroleum-contaminated soils (Schwendinger, 1968).

Variations in chemical composition among different oils might require applying unique mixtures of microorganisms (Buckingham, 1981). Different mixtures of seed microorganisms might also be necessary for stimulated oil biodegradation in different soil environments (Westlake, Jobson, Phillippe, and Cook, 1974). Some compounds may be highly resistant to microbial attack, or recalcitrant, including the high molecular weight and highly branched and condensed ring compounds found in many oils (Atlas and Bartha, 1973b). These would be left as a tar-like residue that would not be easily biodegradable.

Microbial inoculants covering a broad range of metabolic capabilities are available commercially, and they are being used increasingly in treating both contaminated soil and aquatic systems (Thibault and Elliott, 1980). Table C-5 lists a few suppliers and their microbial augmentation products or processes for treatment of hazardous waste-contaminated soils. Appendix A, Section 5.1.1.1, provides additional information on using seed organisms for biodegradation.

Commercially available mutant bacteria generally require an adaptation period in order to adjust to site conditions (Buckingham, 1981). However, the time required is similar to the adjustment period for natural in situ bacteria. Therefore, additional factors, such as convenience and costs of application and labor must be considered in selecting the most efficient bacterial treatment.

When groundwater is treated with added bacterial strains, the contaminated water is often pumped from the ground from one or more wells to a tank where aeration and nutrient addition occur (Thibault and Elliott, 1979). The organisms can conveniently be added with the nutrient solution. The water is then pumped back into the ground at a point "upstream," relative to the groundwater flow. Thus, a closed loop for biological treatment is developed. Depending upon the tank configuration, a number of mechanical or diffused aeration devices are available commercially.

Inoculation of contaminated soil with microorganisms selected for their ability to degrade or transform hazardous materials is a very attractive treatment concept (JRB Associates, Inc., 1984b). However, the treatment manager must realize that the soil environment is restrictive and the soil microbial

system has a complex ecology. These factors may limit the ability of introduced organisms to become self-perpetuating and carry out their specialized functions for an extended period of time. It may be necessary to reinoculate the soil several times before satisfactory levels of treatment are achieved. Cost must also be considered, and there is no point in adding microorganisms, if seeding is not necessary. Even if microbial seeding is used, it will generally have to be accompanied by environmental modifications (Atlas, 1977). Seeding without simultaneous modification of environmental conditions is not likely to succeed.

In most ecosystems, some environmental factor will limit oil-degrading activity (Atlas, 1977). The application of microbial amendments to the soils is frequently combined with other treatment techniques, such as soil moisture management, aeration, and fertilizer addition (Thibault and Elliott, 1980). This method may be most effective against one compound or closely related compounds. The effectiveness would be limited by toxicity of a contaminant or the inability of an organism to metabolize a wide range of substrates.

5.1.1.1.1 Commensals

When a particular chemical is not easily degraded or is only transformed by microorganisms using it as a sole source of carbon, it is sometimes possible to employ commensalism to encourage complete biodegradation (de Klerk and van der linden, 1974). Bacterial mixtures, such as Pseudomonas, Nocardia, and Arthrobacter, have been used as part of a "cocktail" that may consist of 12 to 20 strains of microbes for treating different types of wastes (Cooke and Bluestone, 1986). The mixture of organisms depends upon the composition of the waste. Both selective and controlled mixed cultures are currently being used (Pfennig, 1978a). Specific organisms for target chemicals can be chosen. For example, purple nonsulfur bacteria are used to remove organic compounds and green sulfur bacteria to remove hydrogen sulfide.

5.1.1.1.2 Acclimated Microorganisms

The adaptation process during which microorganisms adjust to growing on a new substrate is defined functionally as an increase in rate of degradation with exposure to a compound (Swindoll, Aelion, Dobbins, Jiang, Long, and Pfaender, 1988). It may involve one or more of the following: 1) an induction or derepression of enzymes specific for the degradation pathways of a particular compound, 2) an increase in the number of organisms in the degrading population, 3) a random mutation in which new metabolic pathways are produced that will allow degradation (Spain, Pritchard, and Bourquin, 1980), or 4) adaptation of existing catabolic enzymes (including associated processes, such as transport and regulatory mechanisms) to the degradation of novel compounds (Harder, 1981).

One approach to biodegradation is the addition to the system of microorganisms that have been especially acclimated to degrade a pollutant of concern (Wilson, Leach, Henson, and Jones, 1986). The organisms may be selected by enrichment culturing or genetic manipulation and can become acclimated to the degradation of compounds by repeated exposure to that substance (Lee and Ward, 1986). Inoculating acclimated organisms into a new environment has met with variable success (Lee, Wilson, and Ward, 1986; Goldstein, Mallory, and Alexander, 1985). There is considerable evidence that this technique is suc-

cessful in degrading a variety of organic contaminants in surface biological waste treatment processes. However, there is less evidence for its application in *in situ* and groundwater environments, mainly because the cases reported were not managed under controlled conditions to differentiate the effects of the acclimated microbes from those of an enhanced indigenous population.

For this approach to be successful in subsurface environments (Wilson, Leach, Henson, and Jones, 1986; Goldstein, Mallory, and Alexander, 1985):

1. The added microorganisms must be able to survive in what to them is a foreign, hostile environment and compete for nutrients with indigenous organisms.

2. The added microorganisms must be able to move from a point of injection to the location of the contaminant in a medium where bacterial transport is rates are normally very low, especially in fine-grained materials.

3. The added microorganisms must be able to retain their selectivity for metabolizing compounds for which they were initially adapted.

Adaptation affects biodegradation rates (Fournier, Codaccioni, and Soulas, 1981). The rate of transformation of a compound is often increased by prior exposure to the chemical. It can involve different mechanisms, such as gene transfer or mutation, enzyme induction, and population changes. There is a high degree of variability in the ability of a microbial community to adapt, depending upon many factors present at the site (Spain and Van Veld, 1983). Adaptation of aquatic microbial communities can last for several weeks after exposure to a xenobiotic compound. Repeated exposure to a contaminant at a site will usually increase the adaptive capabilities of the microorganisms. In fact, organisms from contaminated sites may be able to degrade a wider range of compounds, once they become acclimated to the organic compounds, a process that may require several months. The ability of communities to adapt varies from site to site. It has been suggested that preadaptation of the microbial population is not a major factor in long-term degradation of petroleum in soils, because adaptation will eventually occur naturally. However, the addition of soil containing previously adapted microorganisms may improve the initial degradation rates (Hornick, Fisher, and Paolini, 1983).

Organisms capable of degrading a recalcitrant contaminant are frequently found in a soil that has been exposed to that contaminant for several years (Brubaker and O'Neill, 1982). This local microbial community is often enriched in enzymes, which allows it to degrade the contaminant, although not necessarily as a sole source of carbon and energy.

Often an organism that is adapted to metabolize a member of a homologous series of molecules may be capable of degrading the rest of the members of the series (Kaufman, 1983). For example, phenol and benzoic acid have been degraded rapidly after acclimatization to *p*-hydroxybenzoic acid (Healy and Young, 1979). Microorganisms adapted to growth on *m*-xylene in the absence of molecular oxygen, with nitrate as an electron acceptor, are also able to degrade toluene under the denitrifying conditions (Zeyer, Kuhn, and Schwarzenback, 1986). Sometimes the compounds are not similar in structure. For instance, after growth with succinate plus biphenyl, a mutant strain of

Beijerinckia could oxidize benzo(a)pyrene and benzo(a)anthracene (Gibson and Mahadevan, 1975). Chemical analog adaptation can reduce the amount of time required for adaptation to a particular chemical.

Appendix A, Section 5.1.1.1.2, suggests procedures that should be used for biodegradation employing acclimated microorganisms and provides examples of the application of this technique in the field.

5.1.1.1.3 Mutant Microorganisms

The inability of a microorganism to degrade a certain substrate may simply be related to its inability to induce certain enzymes or to transport the compound into the cell (Hornick, Fisher, and Paolini, 1983). The provision of microbial strains that exhibit improved biodegradation capacities is one of the most challenging fields of microbiological research related to pollution control (Leisinger, 1983). Strains with improved degradation rates or with a widened range of degradative ability may be constructed by *in vivo* or *in vitro* genetic manipulation.

The design and construction of microbial systems (cellular or extracellular enzymatic) to degrade specific compounds has been made possible by two developments (Pierce, 1982a). First, the ability of microorganisms to degrade some of these compounds has been shown to be encoded on extrachromosomal DNA (plasmids), and, secondly, recent developments in recombinant DNA technology permit the "engineering" of DNA that codes for desired enzymatic capabilities.

Development of mutant strains through genetic alteration may increase growth rate and/or endow the organism with the desired biochemical capability (Thibault and Elliot, 1980a). The addition of adapted and/or mutant microbes has not been completely successful, but has great potential (Fox, 1985). Researchers at Government and industrial laboratories are developing artificially mutated bacteria that will be more active and selective for the chemicals they destroy (Chowdhury, Parkinson, and Rhein, 1986). A number of companies are producing microbial strains to be used to treat abandoned hazardous waste sites and chemical spills (Anonymous, 1981). Adaptation and mutation of microorganisms involves several steps (Zitrides, 1983). Wild strains known to degrade a specific organic chemical or functional group are exposed to successively increasing concentrations of that chemical. Those least inhibited by high concentrations will grow the fastest. These are then irradiated to induce genetic changes for increased growth rate. Although constitutive enzyme systems are being developed to shorten the lag period, both natural *in situ* bacteria and commercial mutant strains require an adaptation period to adjust to site conditions, other factors, such as convenience and costs, must be considered in selecting the most efficient bacterial treatment (Amdurer, Fellman, and Abdelhamid, 1985).

Genetic Engineering

Genetic engineering can be used to increase the degradation capacity of microbes by improving the stability of the enzyme systems (located on chromosomes instead of on plasmids), enhancing their activity, providing them with multiple degradative activities, and ensuring that they are safe, both to the environment and human health (Pierce, 1982d).

Since genes for some enzymes involved in degrading alkanes and simple aromatic hydrocarbons can be found on extrachromosomal DNA elements in the bacterial cell (plasmids) (Alexander, 1981; Hou, 1982; Chakrabarty, 1974; Jain and Sayler, 1987), it may be possible to exchange enzymatic activity among closely related microorganisms through plasmid transfer. Multiple plasmid transfer has been accomplished between Pseudomonas species (Chakrabarty, 1974) to construct a strain that can degrade several hydrocarbons, including octane and naphthalene (Hornick, Fisher, and Paolini, 1983). However, genetic information for enzymes involved in the degradation of many hydrocarbons may not be located on plasmids (Atlas, 1977).

Catabolic plasmids in microorganisms occur naturally and some of the common and well-studied catabolic plasmids are listed in Table A.5-1. By manipulating the exchange of this genetic material, it is possible to develop strains with extended degradative capability; i.e., organisms that can degrade more than one xenobiotic substrate or that can completely mineralize highly recalcitrant molecules (Kamp and Chakrabarty, 1979). The plasmids can be fused together to provide multiple degradative traits or to produce a novel or previously unexpressed degradative pathway (Pierce, 1982d). There is some indirect evidence that this may occur in nature and that plasmid-born genes may be transferred, to some extent, among indigenous bacteria in the soil (Pemberton, Corney, and Don, 1979). It is not known whether introduced microbes would also transfer these genes (Stotsky and Krasovsky, 1981). Stable strains can be engineered that will not pass on their plasmids (plasmids added to stable chromosomes) or will be capable of growth only under restricted conditions; this should limit their potential for escape into the environment. However, environmental conditions and competition with the native microbial population may prevent the genetically engineered organism from reaching its degradative potential.

Genetic engineering can be used to develop organisms with unique metabolic capabilities. Expanding the ability to degrade a wide range of aliphatic and aromatic compounds should give such strains a selective advantage over other bacteria in degrading a mixture of petroleum hydrocarbons (Hornick, Fisher, and Paolini, 1983). However, there is no conclusive evidence that such organisms have been able to establish themselves in aeration basins or in natural environments having an active microbial population (Johnston and Robinson, 1982). The adapted mutant will probably be at a disadvantage in the competition with the native microbial population, and may only be able to proliferate on the substrate upon which it was isolated (Zitrides, 1978; McDowell, Bourgeois, and Zitrides, 1980). Acclimated or genetically engineered organisms may not survive or offer significant advantage in treatment of hazardous wastes unless environmental parameters (oxygen, temperature, nutrients, etc.) can be controlled to promote survival of the added organisms (Fox, 1985).

There are four major areas that may benefit from genetic engineering: stabilization; enhanced activity; multiple degradative activities; and health, safety, and environmental concerns (see Appendix A, Section 5.1.1.1.3) (Pierce, 1982d). Genetic engineering must be used in combination with knowledge of microbial physiology and biochemistry, chemical engineering, and process engineering to develop a successful microbial system that will degrade hazardous organic compounds. Genetic engineering involves implementation of recombinant-DNA procedures and techniques for strain improvement, such as

classical mutation of wild type strains and in vivo gene manipulation techniques (e.g., protoplast fusion, transformation, conjugation, and transduction).

It is important to be able to detect and monitor the fate of genes of a released or introduced organism within a microbial community (Jain and Sayler, 1987). Monitoring of microorganisms is required to predict whether the released organisms would disappear quickly, whether they are unable to compete with the natural microbial community, whether they would proliferate and become temporarily or permanently established in the area, or whether they are being transported from the initial site of the release. Because of the concern over release of genetically engineered organisms into the environment, it may be some time before the issue is resolved and the use of these microbes to clean up hazardous waste sites is widespread (Fox, 1985).

Appendix A, Section 5.1.1.1.3, discusses the methods of producing genetic variants for biodegradation, the advantages of using genetically engineered organisms, the properties of strains that have already been developed (including those with multiple degradative abilities), and the plasmids responsible for degradation of specific hydrocarbons.

5.1.1.2 Use of Analog Enrichment for Cometabolism

Cometabolism of a hazardous compound can be brought about by adding a chemical analog of the compound to contaminated soil or to culture media (Sims and Overcash, 1981). This stimulates production of an enzyme that originally targeted an energy-yielding substrate, but which may also be able to transform the recalcitrant molecule (Alexander, 1981). It has been suggested that a concentration threshold exists for some compounds below which the material cannot be reduced by bacterial action (Bouwer and McCarty, 1984). However, in the presence of one compound at a relatively high concentration, termed the primary substrate, another compound present at trace concentrations, termed the secondary substrate, can also be biotransformed, if the organisms are able to transform both substrates.

Incorporating a small amount of an inducer chemical, to which an organism has become adapted, into a solution of a more persistent compound has been shown to facilitate a more rapid degradation of the compound (Kaufman, 1983). Consideration of the use of inducer molecules to enhance microbial degradation must, however, be accompanied by an understanding of both the mechanisms involved and the ecological acceptability of adding the inducing substrate.

An example of analog enrichment is the use of minimally mutagenic phenanthrene to increase the rate of degradation of highly mutagenic benzo(a)pyrene (Sims and Overcash, 1981). Biphenyl has also been used to stimulate cometabolic degradation of PCBs (Furukawa, 1982). Care must be used in selecting such analogs, since they or their degradation products could be hazardous (Sims and Bass, 1984). They should be added in amounts large enough to stimulate microbial activity, but not at levels that are toxic or that adversely affect public health or the environment. Treatability studies are required to determine the feasibility, loading rate, and effectiveness of the analog(s). The analogs may be applied as solids, liquids, or slurries and mixed thoroughly with the contaminated soil, where feasible. Fertilization may be necessary to maintain microbial activity, and controls may have to be

implemented to prevent drainage and erosion problems. The reliability of this technology is unknown.

When the transformation products from analog enrichment are not hazardous and are degradable by other organisms, this technique may be an effective treatment for contaminated soil (Alexander, 1981). This subject is further discussed in Appendix A, Section 5.1.1.2. Examples of compounds that can be used as analogs for specific hydrocarbons are given in Appendix A, Section 5.1.1.2. Table A.5-2 summarizes a number of organic compounds, their chemical analogs, the organisms that can perform their cooxidation, and the resulting products of the reactions.

5.1.1.3 Application of Cell-free Enzymes

There is evidence for the existence in soil of extracellular enzymes capable of degrading xenobiotics or xenobiotic degradation products (Kaufmann, 1983). Enzymatic degradation of organics with cell-free enzymes holds potential as a possible *in situ* treatment technique (Environmental Protection Agency, 1985b). Purified enzymes, harvested from microbial cells, are commonly used in industry to catalyze a variety of reactions, including the degradation of carbohydrates and proteins.

Microbial enzymes that have the ability to transform hazardous compounds to nonhazardous or more labile products could possibly be harvested from cells grown in mass culture and applied to contaminated soils (Sims and Bass, 1984). Industry commonly employs crude or purified enzyme extracts, in solution or immobilized on glass beads, resins, or fibers, to catalyze a variety of reactions, including the breakdown of carbohydrates and proteins.

There are a number of advantages in using cell-free extracts. Enzyme activity can often be preserved in environments that would be inhospitable to the organisms; e.g., in soils with extremes of pH and temperature, high salinity, or high solvent concentrations. Increased mobility of a compound by extracellular enzymes in sites that inhibit microorganisms may make it more susceptible to decomposition by the soil microflora present in less hostile environments (Munnecke, Johnson, Talbot, and Barik, 1982).

There are also limitations in using the extracts. For an enzyme to function outside the cell in the soil environment, it must not require cofactors or coenzymes. This would limit the application of many enzymes. Most important (especially *in situ*) is enzyme stability--they must be fairly stable in extracellular environments. Enzymes might be leached out of the treatment zone, and they might be inactivated or have lower activity if they are bound to clay or humus in the soil. Outside of biochemical and environmental constraints, logistics and costs for producing enzymes in effective quantities may limit current use of this concept.

Organic wastes are amenable to this treatment. Theoretically, enzymes would quickly transform hazardous compounds if they remained active in the soil, and the potential level of treatment is high. However, there is little information available on the use of this technique in soil and no information from the field. Its reliability is unknown. Further applications and concerns for use of this method are discussed in Appendix A, Section 5.1.1.3.

5.1.1.4 Addition of Antibiotics

Many species of hydrocarbon utilizers have been found to be ingested by a large number of ciliated and other cytophagic protozoans (Texas Research Institute, Inc., 1982). They have been shown to drastically reduce the microbial population, e.g., from 10^7/ml to 10^2/ml.

Protozoa present in sewage can halt the bacterial action on specific pollutants in treatment plants by devouring the bacteria (Anonymous, 1986). Grazing of hydrocarbon-degrading microorganisms by protozoa can lengthen the acclimation period prior to biodegradation in wastewaters (Alexander, 1986). This problem can be eliminated with the use of antibiotics that target the protozoa. With eukaryotic inhibitors (cycloheximide and nystatin) added to untreated sewage, the counts of protozoa can be lowered to 60/ml and the acclimation period reduced from 11 days to 1 day. With no inhibitors, protozoan counts can increase to 3.5×10^4/ml in one day (Wiggins and Alexander, 1986).

5.1.1.5 Effect of Biostimulation on Counts

Application of oily sludge and fertilizer to soil resulted in an increase in colony-forming units (CFUs) (51 percent of total number) but without a simultaneous increase in the direct counts (Lode, 1986). This suggests that many bacteria in soil occur in a state of dormancy, waiting for a suitable material for reactivation (Alexander, 1977). Part of these bacteria might be hydrocarbon degraders, but many are likely to be organisms living on intermediary degradation products.

Addition of nutrients and oxygen to a contaminated site has been shown to effectively increase the number of indigenous microorganisms available for degradation of the contaminants. Appendix A, Section 5.1.1.5, presents examples of counts before and after biostimulation.

Stimulation of pleomorphs (bacteria having multiple forms) in response to adding fertilizer suggests that such organisms adapt to oil degradation more easily than others under improved growth conditions (Lode, 1986). The very high stimulation of nonsporeforming rod-shaped bacteria after sludge and fertilizer application supports the assumption that many of these bacteria (particularly the pigmented types) live on degradation products of hydrocarbons.

5.1.2 Optimization of Soil Factors

The various chemical and physical properties of a soil determine the nature of the environment in which microorganisms are found (Parr, Sikora, and Burge, 1983). In turn, the soil environment affects the composition of the microbiological population both qualitatively and quantitatively. The rate of decomposition of an organic waste depends primarily upon its chemical composition and upon those factors that affect the soil environment. Factors having the greatest effect on microbial growth and activity will have the greatest potential for altering the rate of residue decomposition in soil.

The ability of the upper 6 inches of soil to absorb nutrients and hold water depends upon its physical and chemical properties of texture,

infiltration and permeability, water-holding capacity, bulk density, organic matter content, cation exchange capacity, macronutrient content, salinity, and micronutrient content (Hornick, 1983). A typical mineral soil is composed of approximately 45 percent mineral material (varying proportions of sand, silt, and clay), 25 percent air and 25 percent water (i.e., 50 percent pore space, usually half saturated with water), and 5 percent organic matter, although this is highly variable. Any significant change in the balance of these components could affect the physical and chemical properties of the soil. This may alter the soil's ability to support the chemical and biological reactions necessary to degrade, detoxify, inactivate, or immobilize toxic waste constituents. Appendix A, Section 5.1.2, discusses the effect of waste additions on the physical properties of soil.

Most soils have a tremendous capacity to detoxify organic chemical wastes by diluting the compounds, acting as a buffering system, and decomposing the material through microbial activity (JRB Associates, Inc., 1984b). The most important soil characteristics for this detoxification are those that affect water movement and contaminant mobility, i.e., infiltration and permeability. Certain waste characteristics can also affect soil infiltration and permeability, and this interaction should be taken into account. Table A.5-3 lists the site/soil properties that should be identified to be able to predict potential migration of the contaminating material and indicate what will be necessary for manipulating the soil characteristics for optimum results. Some of the soil factors, however, can be managed only near the surface for enhancing the soil treatment.

Unless all the proper conditions are met for a given compound, biodegradation is not likely to occur (Bitton and Gerba, 1985). Before in situ remedial actions can be initiated for treating hazardous waste-contaminated soils, both the site and waste characteristics must be evaluated (Solanas and Pares, 1984). These features will help determine whether a biological approach is the most feasible treatment option and, if selected, how biodegradation can be used most effectively with the prevailing conditions.

There are more than a thousand different soil types in the United States alone (Federle, Dobbins, Thornton-Manning, and Jones, 1986). The U.S. Soil Conservation Service has characterized certain chemical and physical parameters for many of them while preparing soil maps. These data are readily available. They would help in predicting biomass and activity in various profiles.

The soil environment affects the qualitative and quantitative microbial population (Parr, Sikora, and Burge, 1983). The factors that enhance growth of the microorganisms will also result in more rapid decomposition of the contaminants in the soil. The most important soil factors that affect degradation are water, temperature, soil pH, aeration or oxygen supply, available nutrients (e.g., nitrogen (N), phosphorus (P), potassium (K), sulfur (S)), and soil texture and structure. Any treatments applied to the soil to enhance contaminant removal processes must not alter the physical and chemical environment in such ways that they would severely restrict microbial growth or biochemical activity (Sims and Bass, 1984). In general, this means that the soil water potential should be greater than -15 bars (Sommers, Gilmore, Wildung, and Beck, 1981); the pH should be between 5 and 9 (Atlas and Bartha, 1981; Sommers, Gilmore, Wildung, and Beck, 1981); and the oxidation-reduction (redox) potential should be between pe + pH of 17.5 to 2.7 (Baas Becking, Kaplan, and Moore,

1960). Soil pH and redox boundaries should be carefully monitored when chemical and biological treatments are combined.

Since the activity of microorganisms is so dependent upon soil conditions, modification of soil properties is a viable method of enhancing the microbial activity in the soil (Sims and Bass, 1984). In order to vary these factors for use as a treatment technology, the following information is required:

* Characterization and concentration of wastes, both organics and inorganics, at the site

* Microorganisms present at the site

* Biodegradability of waste constituents (half-life, rate constant)

* Biodegradation products, particularly hazardous products

* Depth, profile, and areal distribution of constituents

* Soil moisture

* Other soil properties for biological activity (pH, Eh, oxygen content, nutrient content, organic matter, temperature)

* Trafficability of soil and site.

The influence of soil factors, such as temperature and nutrient concentration, on phenol mineralization shows great variability as a function of soil type and horizon (Thornton-Manning, Jones, and Federle, 1987). Most of these factors do not function independently; i.e., a change in one may effect a change in others (Parr, Sikora, and Burge, 1983). While the soil factors play an important role in biodegradation, because of these interactions, it is not always easy to predict a priori how temperature or another environmental variable will affect biodegradation in a given soil environment (Thornton-Manning, Jones, adn Federle, 1987). However, if any of the factors that affect degradation processes in soil are at less than an optimum level, microbial activity will be lowered accordingly and substrate decomposition decreased (Parr, Sikora, and Burge, 1983).

Table A.5-6 lists the soil factors that may have to be modified during the use of different treatment technologies: extraction, immobilization, degradation, attenuation, and reduction of volatiles.

5.1.2.1 Soil Moisture

Biodegradation of waste chemicals in the soil requires water for microbial growth and for diffusion of nutrients and by-products during the breakdown process (JRB Associates, Inc., 1984b). A typical soil is about 50 percent pore space and 50 percent solid matter. Water entering the soil fills the pore spaces until they are full. The water then continues to move down into the subsoil, displacing air as it goes. The soil is saturated when it is at its maximum retentive capacity. When water then drains from the pores, the soil becomes unsaturated. Soils with large pores, such as sands, lose water rapidly. If the soil is too impermeable, it will be difficult to circulate treatment

agents or to withdraw the polluted water (Nielsen, 1983). Soils with a mixture of pore sizes, such as loamy soils, hold more water at saturation and lose water more slowly. The density and texture of the soil determine the water-holding capacity, which in turn affects the available oxygen, redox potential, and microbial activity (Parr, Sikora, adn Burge, 1983). The actual microbial species composition of a soil is often dependent upon water availability. The migration of organisms in the soil can also be affected by pore size. Small bacteria are on the order of 0.5 to 1.0 um in diameter (Bitton and Gerba, 1985). Larger bacteria tend to be immobilized in soils by physical straining or filtering.

Field capacity refers to the percentage of water remaining in a soil after having been saturated and free gravitational drainage has ceased (JRB Associates, Inc., 1984b). Gravitational water movement is important for mobilizing contaminants and nutrients, due to leaching. Slow drainage can reduce microbial activity as a result of poor aeration, change in oxidation-reduction potential, change in nutrient status, and increased concentration of natural minerals or contaminants to toxic levels in the pore water. The amount of water held in a soil between field capacity and the permanent wilting point for plants is known as available water. This is the water available for plants and a similar quantity may be required for optimum soil microbial and chemical reactions.

Where it is necessary to predict and interpret the response of microorganisms in soils to organic wastes, both the water content and water potential should be reported (Parr, Sikora, adn Burge, 1983). Water potential is useful for quantifying the energy status of water in soils containing waste chemicals. Generally, with decreasing water potentials, fewer organisms are able to grow and reproduce; and bacterial activity is usually greatest at high water potentials (wet conditions). Species composition of the soil microflora is regulated largely by water availability, which, in turn, is governed essentially by the energy of the water in contact with the soil or waste.

Some fungi can tolerate dry soils and do not grow well if the soil is wet. (Clark, 1967). Bacteria may be antagonistic to fungi under moister conditions. At low potentials, bacteria are less active, allowing fungi to predominate (Cook and Papendick, 1970). Microbial decomposition of organic material in dryer soils is probably due primarily to fungi (Gray, 1978; Harris, 1981). When soil becomes too dry, many microorganisms form spores, cysts, or other resistant forms, while many others are killed by desiccation (JRB Associates, Inc., 1984b). Although fine-textured soils have the maximum total water-holding capacity, medium-textured soils have the maximum available water due to favorable pore size distribution.

A well-drained soil (e.g., a loamy soil) is one in which water is removed readily but not rapidly (JRB Associates, Inc., 1984b). A poorly drained soil (e.g., a poorly structured fine soil) can remain water-logged for extended periods of time, producing reducing conditions and insufficient oxygen for biological activity; an excessively drained soil (e.g., a sandy soil) is one in which water can be removed readily to the point that drought conditions occur. For <u>in situ</u> treatment of hazardous waste-contaminated soils, the most desirable soil would be one in which permeability is only large enough to maximize soil attenuation processes (e.g., adequate aeration for aerobic microbial degradation) while still minimizing leaching.

Control of moisture content of soils at an in-place treatment site may be essential for control and optimization of some degradative and sorptive processes, as well as for suppression of volatilization of some hazardous constituents (Sims and Bass, 1984). The moisture content of soil may be controlled to immobilize constituents in contaminated soils and to allow additional time for accomplishing biological degradation. When contaminants are immobilized by this technique and anaerobic decomposition is desired, anaerobiosis must be achieved by a means other than flooding, such as soil compaction or organic matter addition. Control of soil moisture may be achieved through irrigation, drainage, or a combination of methods. These techniques are discussed in Appendix A, Section 5.1.2.1.

Sometimes a site with shallow depth contamination may require soil mixing to dilute the wastes and incorporate nutrients and oxygen, as well as to enhance soil drying (Sims and Bass, 1984). It may be necessary to install a drainage system to reduce soil moisture. Increasing soil temperature will enhance surface soil drying; this technology, called "Landfarming," is simple and easy to apply. However, drier soil may retard microbial activity, as well as increase volatilization of volatile waste components.

Appendix A, Section 5.1.2.1, supplies additional information on the moisture requirements of organisms and the use of moisture control in biodegradation.

5.1.2.2 Temperature

Soil temperature is one of the more important factors controlling microbiological activity and the rate of organic matter decomposition (Sims and Bass, 1984). Temperatures of both air and soil affect the rate of biological degradation processes in the soil, as well as the soil moisture content (JRB Associates, Inc., 1984b).

Generally, an increase in temperature increases the rate of degradation of organic compounds in soil (JRB Associates, Inc., 1962). This rate usually doubles for every 10^{o}C increase in temperature (Thibault and Elliot, 1980a). Conversely, a lowering of the temperature is associated with a slowing of the microbial growth rate. This property is attributed to a decrease in adsorption with rising temperature, which makes more organics available, or to an increase in biological activity, or both (JRB Associates, Inc., 1984b). Biological activity has an optimum temperature, beyond which biological activity often rapidly decreases, thus displaying a growth curve that is right skewed.

Microbial utilization of hydrocarbons has been shown to occur at temperatures ranging from -2^{o} to 70^{o}C (Texas Reearch Institute, 1982). Biodegradation was found to occur at a temperature of 5^{o}C, but hydrocarbons were degraded more slowly at lower temperatures (Parr, Sikora, and Burge, 1983). Most soils, especially those in cold climates, contain psychrophilic microorganisms that grow best at temperatures below 20^{o}C (JRB Associates, Inc., 1984b) and are effective at temperatures below 0^{o}C. Soils in hot environments usually support many thermophilic microorganisms that are effective at temperatures above 60^{o}C. However, most soil microorganisms are mesophiles and exhibit maximum growth in the range of 20^{o} to 35^{o}C (Parr, Sikora, and Burge, 1983). The majority of hydrocarbon utilizers are most active in this range.

Temperatures in the thermophilic range (50 to 60°C) were shown to greatly accelerate decomposition of organic matter, in general (Parr, Sikora, and Burge, 1983). At these temperatures, actinomycetes will be naturally predominant over fungi and bacteria. Therefore, in certain situations, composting may offer potential for maximizing the biodegradation rate of waste industrial chemicals. It should be noted, however, that in another investigation in a test treatment facility, it was found that several aromatic hydrocarbons were not metabolized at 55°C, but were metabolized at 30°C (Phillips and Brown, 1975), while other researchers reported a leveling-off of the hydrocarbon biodegradation rate in soil above 20°C (Dibble and Bartha, 1979a). Although elevated temperature has some advantage for potentially limiting the development of pathogenic microorganisms, too high a temperature would not be beneficial to stimulate petroleum biodegradation (Phillips and Brown, 1975). The increased availability of more toxic hydrocarbons at higher temperatures may counteract the stimulation of metabolic processes (Dibble and Bartha 1979a).

A microbial community will undergo an adaptation or selection process in the mineralization of a compound, which is reflected in a lag period that often increases with decreasing temperature (Thornton-Manning, Jones, and Federle, 1987). Temperature was also found to affect phenol mineralization differently, as a function of soil type.

The effect of temperature on degradation is important in terms of assessment of the seasonal and geographical variation of degradation rates (JRB Associates, Inc., 1984b). Disposal sites for oil can be chosen in warm areas that receive direct sunlight to assure temperatures suitable for rapid metabolism by mesophilic microorganisms (Atlas, 1977). Even in near-Arctic environments, absorbance of solar energy raises temperatures into a range that allows for mesophilic microbial oil degradation (Atlas and Schofield, 1975).

Soil temperature is difficult to control in a field situation, but can be modified by regulating the incoming and outgoing radiation, or by changing the thermal properties of the soil (Baver, Gardner, and Gardner, 1972). Vegetation plays a significant role in soil temperature because of the insulating properties of plant cover (Sims and Bass, 1984). Bare soil unprotected from the direct rays of the sun becomes very warm during the hottest part of the day, but also loses its heat rapidly at night and during colder seasons. In the winter, the vegetation acts as an insulator to reduce heat lost from the soil. Frost penetration is more rapid and deeper under bare soils than under a vegetative cover. On the other hand, during the summer months, a well-vegetated soil does not become as warm as a bare soil. Fluctuations in soil temperature decrease with increasing depth (Thornton-Manning, Jones, and Federle, 1987).

Appendix A, Section 5.1.2.2, discusses how the thermal properties of soil can be regulated by the use of mulches, irrigation, and compaction. Table C-9 describes many organic substances that can be used as mulch and the specific situations in which each would be most appropriate.

5.1.2.3 Soil pH

Soil pH contributes to the surface charge on many colloidal-sized soil particles (JRB Associate, Inc., 1984b). Clays have a permanent negative charge

and it is primarily their coatings of organic and amorphous inorganic materials that change in charge. At high pH values, the surfaces become more negatively charged; at low pH values, they become positively charged. Thus, the pH of groundwater and soil water in the vadose zone determines the degree of anion or cation adsorption by soil particles. In soils with pH-dependent charge, lowering the pH decreases the net negative charge and, thus, decreases anion repulsion or increases anion adsorption (Hornick, 1983).

Biological activity in the soil is greatly affected by the pH, through the availability of nutrients and toxicants and the tolerance of organisms to pH variations. Some microorganisms can survive within a wide pH range, while others can tolerate only small variations. The optimum pH for rapid decomposition of wastes and residues is usually in the range of 6.5 to 8.5. Bacteria and actinomycetes have pH optima near 7.0. A soil pH of 7.8 should be close to the optimum (Dibble and Bartha, 1979a). If the soil is acidic, these organisms often cannot compete effectively with soil fungi for available nutrients. The pH can influence the solubility or availability of macro- (especially phosphorus) and micronutrients, the mobility of potentially toxic materials, and the reactivity of minerals (e.g., iron or calcium) (Parr, Sikora, and Burge, 1983).

Carbonic acid, organic acid intermediates, and nitrate and sulfate (most important for pH <5), may accumulate during aerobic degradation of organic molecules (Zitrides, 1983). This can lower the soil pH and inhibit biological activity. The acid conditions can, however, be controlled with reinoculation lime addition, or both. In fact, liming has been found to favor the biodegradation of oil (Dibble and Bartha, 1979a).

Hazardous waste-contaminated soil may contain acidic wastes, which will also decrease the soil pH and change the microorganism distribution (Sims and Bass, 1984; JRB Associates, Inc., 1984b). Many fungi predominate under acidic conditions (pH<7). These organisms may transform aromatic hydrocarbons by means of oxygenases into an arene oxide, the mutagenic forms of PAHs (Baver, Gardner, and Gardner, 1972). Bacteria, on the other hand, growing better at a neutral or slightly basic pH, would carry out the dioxygenation of the aromatic nucleus to form a cis-glycol as the first stable intermediate, instead of the arene oxide. Higher organisms (above fungi) do not possess the necessary oxygenases and, thus, form trans aromatic diols, which tend to polymerize.

These differences in the mechanism of aromatic hydrocarbon metabolism by microorganisms have important implications concerning engineering techniques for controlling and possibly detoxifying simple aromatics and PAHs in contaminated soils (Cerniglia, Herbert, Dodge, Szaniszlo, and Gibson, 1979). It appears that selection for dominance of the microbial community by bacteria may avoid the formation of mutagens, and that pH may serve as an important engineering tool to direct the pathway of PAH degradation.

Control of soil pH at an in-place hazardous waste treatment site is a critical factor in several treatment techniques, including metal immobilization and optimum microbial activity (Sims and Bass, 1984). The goal of soil pH adjustment in agricultural application usually is to increase the pH to near neutral values, since most natural soils tend to be slightly acidic, 1984). Areas of the country in which the need for increasing soil pH is greatest are the humid regions of the East, South, Middle West, and Northwest States. In

areas where rainfall is low and leaching is minimal, such as parts of the Great Plain States and the arid, irrigated saline soils of the Southwest, Intermountain, and Far West States, pH adjustment is usually not necessary but may require reduction.

The soil pH may affect the solubility, mobility, and ionized forms of contaminants, (JRB Associates, Inc., 1984b). The pH of different soil types can vary. A calcareous (containing calcium carbonate) soil can range from pH 7 to 8.3. A sodic (high in sodium carbonate) soil can go as high as pH 8.5 to 10. Saline soils tend to be around pH 7. The soil pH may need to be lowered by adjusting with sulfur or other acid-forming compounds, or raised by adding crushed limestone or lime products to bring it between pH 5.5 and 8.5 to encourage microbial activity. Phosphorus solubility is maximized at pH 6.5; this may be the ideal soil pH. See Appendix A, Section 5.1.2.3, for information on methods for increasing and decreasing soil pH.

5.1.2.4 Oxygen Supply

The degree to which the soil pore space is filled with water affects the exchange of gases through the soil (JRB Associates, Inc., 1984b). Microbial respiration, plant root respiration, and the respiration of other organisms removes oxygen from the soil and replaces it with carbon dioxide. Gases slowly diffuse into the soil from the air above, and gases in the soil slowly diffuse into the air. However, the oxygen concentration in surface unsaturated soil may be only half that in air, while carbon dioxide concentrations may be many times that of air (Brady, 1974).

As the soil becomes saturated, the diffusion of gases through the soil is severely restricted. In saturated soil, oxygen can be consumed faster than it can be replaced, and the soil becomes anaerobic (JRB Associates, Inc., 1984b). This drastically alters the composition of the microflora. Facultative anaerobes, which use alternative electron acceptors, such as nitrate (denitrifiers), and strict anaerobic organisms become the dominant species. While many soil bacteria can grow under anaerobic conditions, though less actively, most fungi and actinomycetes do not grow at all (Parr, Sikora, and Burge, 1983). Microbial metabolism shifts from oxidative to fermentative and becomes less efficient in terms of biosynthetic energy production (JRB Associates, Inc., 1984b). Soil structure and texture primarily determine the size of soil pores, and hence the water content at which gas diffusion is significantly limited in a given soil, and the rate at which anaerobiosis sets in.

As the oxygen is depleted from soils, the reactions become anaerobic with the production of malodorous compounds, such as amines, mercaptans, and H_2S (Parr, Sikora, and Burge, 1983). These can be phytotoxic, and if the soil is heavily overloaded, the soil may remain anaerobic for some time. However, if the oxygen balance is maintained, relative to the amount of contaminants and the soil conditions, rapid aerobic decomposition will occur, and the end products will be inorganic carbon, nitrogen, and sulfur compounds.

Several alternative sources of oxygen have been suggested as a means to increase the degradative activity in contaminated aquifers (Texas Research Institute, 1982) (see Appendix A, Section 5.1.2.4). This section of Appendix A also describes the oxygen requirements for biodegradation of organic compounds and summarizes the advantages and disadvantages of various options for

supplying oxygen to the subsurface (Table A.5-7). Oxidizing agents can be used to degrade organic constituents in soil systems, although they may themselves be toxic to microorganisms or may cause the production of more toxic or more mobile oxidation products (Sims and Bass, 1984). Two powerful oxidizing agents for in-place treatment, ozone and hydrogen peroxide, are discussed in depth in Appendix A. Approaches for modifying soil oxygen for both aerobic and anaerobic biodegradation are also described in Appendix A, Section 5.1.2.4.

5.1.2.5 Nutrients

Microbial degradation of hazardous compounds requires the presence of certain nutrients for optimum biological growth (Table A.5-7). Feeding nutrient solutions containing inorganic nutrients, such as soluble nitrogen, phosphorus, and sulfur compounds, to natural soil bacteria often enhances the ability of the microorganisms to degrade organic molecules into carbon dioxide and water (Stotzky and Norman, 1961a; Stotzky and Norman, 1961b).

At one site, decomposition of oil in the soil was shown to proceed at a rate of 0.5 lb/ft^3/month without a nutrient source, and at 1.0 lb/ft^3/month after the addition of fertilizer (Kincannon, 1972). Without added nutrients, aromatic hydrocarbons were noted to be more readily attacked than saturated aliphatic hydrocarbons by the microbes (Atlas, 1981). Addition of nitrogen or phosphorus stimulated degradation of saturated hydrocarbons more than of aromatic hydrocarbons.

These nutrients may be present in contaminating wastes, but may not be readily available or may not supply all that is required (Sims and Bass, 1984). Their supplementation may be necessary. Three of the major nutrients, nitrogen, phosphorus, and potassium, can be supplied with common inorganic fertilizers (JRB Associates, Inc., 1984b). The carbon, nitrogen, and phosphorus content of bacterial cells is generally in the ratio of 100 parts carbon to 15 parts nitrogen to 3 parts phosphorus (Zitrides, 1983). (Alexander, 1977 specifies the approximate C:N:P: ratio found in bacterial biomass as 120:10:1.) By knowing how much of the carbon in a spilled substance ends up as bacterial cells, it is possible to calculate the amount of nitrogen and phosphorus necessary to equal this ratio for bacterial growth (Thibault and Elliot, 1980a). This procedure is explained in Appendix A, Section 5.1.2.5. Sufficient nitrogen and phosphorus should be applied to ensure that these nutrients do not limit microbial activity (Alexander, 1981).

The main danger at hazardous waste sites may be in overloading the soil with elements that may have been present in the waste (or already in the soil, e.g., phosphate plugging), causing toxicity and leaching problems (JRB Associates, Inc., 1984).

Appendix A, Section 5.1.2.5, further discusses nutrient requirements for biodegradation in different environments, the effect of various additives, and precautions that should be observed in adding certain substances. An optimum fertilization plan is proposed.

5.1.2.6 Organic Matter

Organic material is very important in the soil matrix (Hornick, 1983). The presence of organic materials may have many effects on soil properties,

including degree of structure, water-holding capacity, bulk density, mobilization of nutrients (hindering degradation of organic wastes), reduction in soil erosion potential, and soil temperature (Atlas, 1978c). Naturally occurring organic material can influence the ability of microorganisms to degrade pollutants (Shimp and Pfaender, 1984). Its role in metal reactions or sorption processes that occur in the soil determines the availability of metals and essential nutrients for plants and microorganisms (Hornick, 1983). Sorption of contaminants on soil particles can alter the molecular character and enzymatic attack of a given compound. Humic polymers can act as stabilizing agents, making the compounds less resistant to biodegradation (Verma, Martin, and Haider, 1975). However, the opposite effect has also been observed, with bound material becoming unavailable for biodegradation. After adaptation of a microbial community to four types of compounds, it was found that amino acids, fatty acids, and carbohydrates stimulated biodegradation of monosubstituted phenols, while humics decreased biodegradation rates (Shimp and Pfaender, 1984).

Soil contains organic material in varying stages of decomposition (JRB Associates, Inc. 1984b). Around 65 to 75 percent of this material usually consists of humic substances; i.e., humic acid, fulvic acid, and humin, which have very large surface areas and high cation exchange capacities (Schnitzer, 1978). The remainder of the organic material consists of polysaccharides and proteins, such as carbohydrates, proteins, peptides, amino acids, fats, waxes, alkanes, and low molecular weight organic acids, which are rapidly decomposed by the soil microorganisms (Schnitzer, 1982). This organic matter can also contribute nitrogen, phosphorus, sulfur, zinc, and boron, all of which add to the nutrient status of the soil (JRB Associates, Inc., 1984b).

If biodegradable organic materials are added to the soil in order to raise the carbon to nitrogen ratio higher than about 20:1, mineral nitrogen in the soil will be immobilized into microbial biomass, and the decomposition process will be slowed considerably (JRB Associates, Inc., 1984b). Phosphorus is similarly immobilized when carbon is in excess (Alexander, 1977). If the soil must be managed to decompose organic matter during the treatment of hazardous waste-contaminated soils, nitrogen and phosphorus may be required to bring the C:N:P ratio close to that of the bacterial biomass. However, C:N ratios should be used cautiously, since they do not indicate the availability of the carbon or nitrogen to microorganisms (Taylor, Parr, Sikora, and Willson, 1980).

Organic matter is very important to the microbial ecology and activity of the soil (Sims and Bass; JRB Associates, Inc., 1984b). Its high cation-exchange capacity and high density of reactive functional groups help to bind both organic and inorganic compounds that may be added to the soil. These properties also help to retain the soil bacteria which can then attack the bound compounds. Thus, the sorbents may immobilize the organic constituents, as well as allow more time for biodegradation. Microbial enzymes can sometimes catalyze the binding of xenobiotics to soil humus, where local conditions may regulate their subsequent release (de Klerk and van der linden, 1974). If the soil contains cracks and fractures that may increase the potential for mobilization and groundwater contamination, addition of an adsorbent can be useful (Sims and Bass, 1984; JRB Associates, Inc., 1984b). It is especially important and effective in soils with low organic matter content, such as sandy and strip-mined soils. These sorbants include agricultural products and by-products, sewage sludges, other organic matter, and activated carbon.

Supplemental carbon and energy sources can be used to stimulate the metabolism of even recalcitrant xenobiotics, either through cometabolism (Alexander, 1981) or simply because of the presence of additional carbon and energy (Yagi and Sudo, 1980). The naturally occurring organic components of soil and their involvement in biodegradation are further discussed in Appendix A, Section 5.1.2.6.

5.1.2.7 Oxidation-reduction Potential

The oxidation-reduction potential, or Eh, of the soil in question basically expresses the electron availability as it affects the oxidation states of hydrogen, carbon, nitrogen, oxygen, sulfur, manganese, iron, cobalt, copper, and other elements with multiple electron states in aqueous systems (Bohn, 1971). This indicates the electron density of a system. As a system becomes reduced, there is an increase in electron density and negative potential (Taylor, Parr, Sikora, and Willson, 1980). Eh decreases during flooding and increases during drying (Bouwer, 1984). The fastest changes occur within the top two centimeters.

The degradative pathways for some hazardous compounds may involve reductive steps (JRB Associates, Inc., 1984b). This may occur as an initial reaction that requires anaerobiosis, or it may be expressed by more rapid degradation under anaerobic conditions. Many compounds, such as heavily chlorinated compounds, can be transformed under anaerobic or alternating anaerobic-aerobic conditions, but not readily under strict aerobic conditions (Wilson and Wilson, 1985). As previously stated, the initial dechlorinations occur most readily under anaerobic conditions, with chlorine acting probably as an electron acceptor. Some contaminated aquifers will be anaerobic, and if the microbial population is capable of degrading the material, it may be possible to use anaerobic *in situ* techniques to treat some compounds.

The redox conditions can be controlled to achieve conditions under which specific compounds can be degraded, dehalogenated, or particular organisms or enzyme systems can be selected (Wilson and Wilson, 1985). Alteration of aerobic/anaerobic conditions by adjusting Eh through flooding or cultivation can, therefore, be a useful tool for engineering management to maximize detoxification and degradation of some compounds (Guenther, 1975). Anaerobic conditions can be maintained by keeping the soil saturated with water and limiting aeration. Regular cultivation of soil should maintain aerobic conditions. Table A.5-8 shows the succession of events related to the redox potential, which can occur in poorly aerated soils receiving excessive loadings of organic material.

There are economic advantages of using anaerobic degradative processes. For example, accentuated anaerobic metabolism of fuel oil components would eliminate problems (plugging, intensive management) caused by hydrogen peroxide addition to wells. Although pertinent mainly to chlorinated solvents and PCBs (not really addressed in this report), anaerobic conditions are required to dehalogenate some highly chlorinated materials (e.g., tetrachloroethylene and perhaps higher chlorinated PCBs), while aerobic conditions seem to be required to remove halogens from lower chlorinated compounds (dichloroethylene, vinyl chloride). Thus, anaerobic-aerobic control may be of value here. Stanford University is investigating accentuation of the anaerobic breakdown of aromatic compounds, because the process may be cost-effective.

Appendix A, Section 5.1.2.7, presents different ways to express the oxygen levels in soils and aquatic surface and subsurface environments. It also classifies oxygen levels in various soil types, based upon their redox potential.

5.1.2.8 <u>Attenuation</u>

The basic principle of attenuation is the mixing of contaminated soil (or wastes) with clean soil to reduce the concentrations of hazardous compounds to acceptable levels (Sims and Bass, 1984). This is applicable to both inorganics and organics. The mixing of uncontaminated soil with the contaminated increases the extent and effectiveness of immobilization of chemical contaminants and may also aid in decreasing toxicity of the contaminated soil to the soil microorganisms involved in biodegradation. In practice, attenuation systems have been designed, and acceptable concentration limits established, only for heavy metals. However, in principle, this technique should also apply to organic contaminants.

Attenuation is included in Table A.5-4, which lists the soil modification requirements for several treatment technologies. See Appendix A for further discussion of this practice.

5.1.2.9 <u>Texture and Structure</u>

Soil type may be an important determinant of whether groundwater pollutants are biodegraded as they pass through the unsaturated zone of a soil profile (Federle, Dobbins, Thornton-Manning, and Jones, 1986). Microorganisms have been shown to be present, often in large numbers, in the entire vertical profiles of sediments in wells several hundred feet deep. The vertical distribution of microorganisms in a soil profile differs greatly as a function of soil type. In four different soil types, biomass and activity declined with increasing depth; however, the magnitude and pattern of this decline differed for each soil type (see Table A.3-3). The soil type also affected the types of microbial populations present. Horizon and soil type affect the time of transit of a contaminant, as well as the potential for biodegradation. The type of soil will also influence the mobility of microorganisms through the subsurface. Bacteria generally do not move large distances in fine-textured soil (less than a few meters, for example), but they can travel much larger distances in coarse-textured or fractured materials (Romero, 1970).

The composition of soil influences infiltration rate and permeability, water-holding capacity, and adsorption capacity for waste components (Hornick, 1983). These, in turn, have an effect on the biodegradability of the contaminating wastes and the ability of microorganisms to metabolize the compounds. Appendix A, Section 5.1.2.9, describes how the physical properties of soil (i.e., texture, bulk density, and water-holding capacity) function in the process of biodegradation.

Bacteria generally do not move large distances in fine-textured soil (less than a few meters, for example), but they can travel much larger distances in coarse-textured or fractured materials (Romero, 1970).

5.1.3 Alteration of Organic Contaminants

When the concentration or availability of organic substrate is low, increasing the surface area-to-liquid volume ratio improves substrate utilization and bacterial growth rates (Heukelekian and Heller, 1940; Characklis, 1973).

5.1.3.1 Addition of Surfactants

Surface active agents are a class of natural and synthetic chemicals that promote the wetting, solubilization, and emulsification of various types of organic chemicals (Amdurer, Fellman, and Abdelhamid, 1985). Surfactants are organic molecules and can be either cationic, anionic, or nonionic (JRB Associates, Inc., 1984b). These agents would be most effective in promoting the mobilization of organic compounds of relatively low water solubility and high lipid solubility (high K_{ow} values). Surfactants may enhance the recovery of subsurface gasoline contamination by groundwater pumping and promote the mobilization of hydrophobic contaminants from unsaturated soils (Ellis and Payne, 1984).

The physical and biological properties of soil may be affected when surfactants are added (Ellis and Payne, 1984). A concentration of 100 ppm of most synthetic emulsifiers is not substantially deleterious to microorganisms. However, detergents and other strong (especially synthetic) emulsifiers/surfactants may disrupt cell membranes (lipids) or cause toxicity by excessive uptake of toxic hydrophobic contaminants. In addition, surfactants may be precipitated by groundwater with high TDS or alkaline earth cation concentrations (Ca, Mg). They require optimal pH and temperature and can be adsorbed by soil particles, negating their solubilizing properties. Nevertheless, use of surfactants alone to flush otherwise insoluble organics, or in combination with other treatments to solubilize waste materials (thereby, promoting biodegradation), is a promising avenue for further research.

Microbes have been long known to produce surface active agents when grown on specific substrates (Zajic and Panchal, 1976). Many of these surfactants have excellent emulsifying properties (Panchal and Zajic, 1978). Many oil-degrading microorganisms produce emulsifying agents (Reisfeld, Rosenberg, and Gutnick, 1972), and naturally occurring biosurfactants seem to be very important in the elimination of hydrocarbons from polluted biotopes (Rambeloarisoa, Rontani, Giusti, Duvnjak, and Bertrand, 1984). These biosurfactants contain sugars, fatty acids, and lipids (mono- and diglycerides) and have a strong emulsifying effect on petroleum contaminants. Attempts have been made to isolate these emulsifying agents for possible use in dispersing oil. Bioemulsifiers are generally easy and inexpensive to produce and are usually nontoxic (Zajic and Gerson, 1977). Production of these compounds can be easily controlled by media manipulation, and emulsifiers of biological origin are being applied for industrial purposes (Zajic and Panchal, 1976).

Microorganisms generally consume only soluble or solubilized (emulsified) organic molecules; and synthesis of an emulsifier may pseudosolubilize target hydrocarbons (Thibault and Elliot, 1980a; Goma, Al Ani, and Pareilleux, 1976). An _Arthrobacter_ strain was found to extensively emulsify oil when growing on hydrocarbons (Reisfel, Rosenberg, and Gutnick, 1972). However, organisms that can emulsify oil often do not extensively degrade the hydrocarbons in the oil

(Atlas, 1981). Where the hydrocarbonoclastic microbes do not have strong emulsification ability, commercially produced, biodegradable emulsifiers could be added to accelerate the biodegradation process. In fact, biodegradation rates could perhaps be enhanced by use of microbes that secrete large quantities of such surface-active agents (Zitrides, 1983). Appendix A, Section 5.1.3.1, describes how microorganism-generated or externally added surface active agents effect the uptake of substances microorganisms could not previously utilize.

There are also many chemical oil dispersants that could be used for this purpose (Canevari, 1971). Successful chemical enhancement of oil biodegradation is dependent upon the particular emulsifying agent (Robichaux and Myrick, 1972). The problem with dispersing oil, even if it accelerates soil biodegradation, is that many oil dispersants can be toxic to soil organisms (Shelton, 1971). On the other hand, some emulsifying agents may not produce these undesirable effects.

Both an oil and a water phase are present in soil contaminated with petroleum products (Genner and Hill, 1981). Nutrients, inhibitors, and metabolic products are always partitioned between these phases. Microorganisms proliferate in the water phase and migrate into the hydrocarbon phase, where they usually die. Hydrocarbon-degrading microorganisms are found mainly at the oil-water interface (Atlas, 1981). In soil, microbes break down oil at oil-water interfaces (Flowers, Pulford, and Duncan, 1984). These organisms can be seen growing over the entire surface of an oil droplet. Growth does not appear to occur within oil droplets in the absence of entrained water. Increasing the oil surface area should accelerate biodegradation. The material is more readily available to microorganisms, and movement of emulsion droplets through the water column increases the availability of oxygen and nutrients.

This led to the commercial development and application of synthetic biodegradable emulsifiers for the cleanup of land-based spills of hydrophobic materials, such as crude and refined petroleum products (Thibault and Elliott, 1979). These emulsifiers act rapidly on the hydrocarbons and, therefore, assist in their rapid assimilation by the indigenous microorganisms. Use of mutant microorganisms that produce large quantities of biosurfactants have also been shown to be useful in accentuating petroleum hydrocarbon degradation.

Bioemulsifiers are highly substrate specific and are most effective with mixtures of compounds, which may not be emulsified individually (Rosenberg, Zuckerberg, Rubinovitz, and Gutnick, 1979). Bioemulsifiers and biosurfactants may enhance pseudosolubilization of hydrocarbons or direct contact between microorganisms and a hydrocarbon substrate. It should also be noted that emulsification of the hydrocarbons will aid in mobilizing them through the soil (Vanloocke, Verlinde, Verstraete, and DeBurger, 1979).

See Appendix A, Section 5.1.3.1, for a discussion of the application of bioemulsification to petroleum product spills.

5.1.3.2 Supplementing Threshold Concentrations of Contaminants

Laboratory evidence clearly indicates that under proper conditions, many potentially hazardous organic compounds can be biodegraded in the ground, even when present at very low concentrations (Bitton and Gerba, 1985). However,

evidence suggests that there may be a threshold concentration for a given contaminant, below which it will not be biodegraded. This would be expected, if the energy derived from low concentrations of the substrate was inadequate for maintenance of the bacterial cell, or if higher concentrations were required to activate the transport and metabolic systems of the cell (Boethling and Alexander, 1979). By adding more of the given contaminant (or other less or nonhazardous analogs) this threshold could perhaps be exceeded, since more inducible enzyme(s) would be produced (for example, adding fulvic acid to stimulate the degradation of benzene, toluene, or phenols).

5.2 OPTIMIZATION OF GROUNDWATER BIODEGRADATION

Some of the information in this section may duplicate material covered in Section 5.1, Optimization of Soil Biodegradation, and Section 5.3, Optimization of Freshwater, Estaurine, and Marine Biodegradation; however, it is presented here under a separate heading, with other related information, to accommodate those readers who may specifically wish to address treatment of groundwater contamination only.

Materials from a spill normally reach a groundwater system either through direct contact with a recharge area or by rainwater percolating through contaminated soil (Buckingham, 1981). Examination of these mechanisms, as well as practical sampling, testing, and management techniques, can make it possible to treat a wide variety of spills through the use of a biodegradation scheme.

Microbial activity in aquifers may be limited by the levels of dissolved oxygen, nutrients, and the numbers of microorganisms capable of degrading the contaminants (Lee and Ward, 1983). In situ treatment processes usually involve the circulation of both oxygen and inorganic nutrients through the aquifer so indigenous organisms can degrade the contaminants (Lee and Ward, 1985). Supplying dissolved oxygen to the groundwater is likely to be the limiting factor in the biostimulation process, especially in low-permeability aquifers.

If a contaminated water table aquifer is oxygenated and contains organisms capable of degrading petroleum hydrocarbons, there is potential for natural remediation of the plumes with time (Lee and Ward, 1984a; Wilson and Rees, 1985). When an aquifer is anaerobic and there is no significant degradation activity after a gasoline or fuel oil contamination, oxygen and nutrients can be added, with a resulting rapid degradation of the hydrocarbons down to approximately 1 ug/l with little lag time (Josephson, 1983b). This, however, requires an initial hydrocarbon concentration below about 10 mg/l. Higher concentrations may produce a considerable adaptation time.

Withdrawal and biological treatment seem to currently be the most effective methods for biological restoration of aquifers contaminated by organic compounds (Knox, Canter, Kincannon, Stover, and Ward, 1968). Biostimulation by the addition of oxygen and nutrients has been chiefly used to reclaim aquifers contaminated by gasoline and has been effective in reducing, although not completely eliminating, the quantity of gasoline. Enhancing the indigenous microbial population has also been fairly effective in above-ground treatment of groundwater contaminated with organic solvents, but augmentation has generally failed to increase petroleum hydrocarbon degradation rates. Seeding contaminated sites with microorganisms and the use of ozone or hydrogen peroxide to increase in situ oxygen levels are promising developments but are, as of yet, unproved.

Biodegradation of contaminants by the indigenous microbial population is one of the most effective methods for removing organic contamination. Manipulating these variables may help improve the biodegradation.

5.2.1 Biological Enhancement

Microorganisms in the groundwater can transform many of the organic contaminants typically found in contamination incidents (Wilson, Leach, Henson,

and Jones, 1986). Different isolates are able to directly utilize certain components of gasoline, while cooxidation plays an important role in the biodegradation of the other compounds (114b/e). Aquifer sediment microorganisms can mineralize trace levels of petroleum hydrocarbon compounds, and the rates can be affected by preexposure of the microbial community to the petroleum product (Dooley, Larson, and Ventullo, 1985).

An approach for *in situ* treatment of contaminated aquifers is to promote a particular microbial population with specific metabolic capabilities to flourish. (Lee and Ward, 1986).

5.2.1.1 Seeding of Microorganisms

Maintenance of the bacterial population at optimal levels is important, especially for selective mutant organisms, which tend to be more sensitive to environmental variables than naturally occurring species (Environmental Protection Agency, 1985b). In theory, a continuous incubation facility operating at optimal growth temperatures and under more controlled conditions could be used to maintain the microbial population. The high biomass-containing stream produced from such a facility could then be reinjected *via* wells or trenches so as to reinoculate the subsurface continuously with microorganisms, although clogging of the soil and transport could be serious problems. It has been suggested that seed bacteria in an aqueous environment be encapsulated to ensure they adhere to and remain with the contaminating oil (Gholson, Guire, and Friede, 1972).

5.2.1.2 Acclimation

Organisms from contaminated sites may be capable of degrading a wider range of compounds once they become acclimated to the organic compounds, a process that may require several months (Wilson, McNabb, Cochran, Wang, Tomson, and Bedient, 1986) or even years (Mackay, Roberts, and Cherry, 1985). Other authors reported a lag period of one to five days, depending upon temperature, before the groundwater microbial flora are able to measurably degrade dissolved hydrocarbons (Kappler and Wuhrman, 1978a; Kappler and Wuhrman, 1978b). The biotransformation rates of trace organic contaminants are highly variable, with half-lives ranging from a few days to many years. Use of microorganisms preadapted to degrade specific chemicals can reduce the lag period normally required for acclimation to the substrates (Mackay, Roberts, and Cherry, 1985). Previously acclimated organisms have been employed in remedial actions to clean up contaminated groundwater (Lee and Ward, 1986).

Withdrawal and biological treatment of contaminated groundwater has been shown to be an effective, albeit slow, method for restoration of aquifers and improvement of degradation rates (Ward and Lee, 1984). Activated sludge treatment of pumped groundwater, often in conjunction with physical treatment processes (such as activated carbon adsorption or air stripping), has been demonstrated to build up an acclimated microbial population that can degrade most petroleum contaminants. See Appendix A, Section 5.2.1.2, for a further discussion of acclimation of microorganisms in aquifer restoration.

5.2.2 Optimization of Groundwater Factors

The biodegradation of a particular class of organic contaminants depends upon the physiological capabilities of the organisms in the aquifer (Wilson, Leach, Henson, and Jones, 1986). These capabilities depend, in turn, upon the geochemical environment of the organisms. Essentially, degradation of organic compounds in groundwater depends upon the geochemical properties of the groundwaters that receive them.

Many of the same procedures for supplementing aquatic and soil systems can be used to restore contaminated aquifers (Ward and Lee, 1984). Contaminated groundwater can be pumped to the surface where aeration, nutrients, microorganisms, and surfactants can be added before it is reinjected (Zitrides, 1983). In addition, it is possible to modify the groundwater (Lee and Ward, 1986). A pilot study where nutrients and oxygen were supplied to a contaminated aquifer showed that the microbial population increased from 10^3/ml to 10^6/ml after seven days and remained constant at that level (Knox, Canter, Kincannon, Stover, and Ward, 1968). Pumping and treating water from a contaminated aquifer, by using activating tanks, maintaining the temperature at 20°C, adding air and nutrients, and supplying an acclimated bacterial culture, resulted in reducing the concentration of the contaminants to an acceptable level in a year.

5.2.2.1 Temperature

As in soil, temperature plays a significant role in aquatic systems. The temperature range for optimal organism growth in aerobic biological wastewater treatment processes has been found to range from 20° to 37°C (68° to 99°F) (Environmental Protection Agency, 1985b). According to the "Q-10" rule, for every 10°C decrease in temperature in a specific system, enzyme activity is halved. Figure A.5-3 provides typical groundwater temperatures throughout the United States.

Raising the temperature of a contaminated zone by pumping in heated water or recirculating groundwater through a surface heating unit may be feasible under conditions of low groundwater flow (Environmental Protection Agency, 1985b).

5.2.2.2 Oxygen Supply

At many sites contaminated with petroleum hydrocarbon contaminants, the levels of dissolved oxygen may control biodegradation (Lee, Thomas, and Ward, 1985). For biodegradation of most organic contaminants, roughly two parts of oxygen are required to completely metabolize one part of organic compound (Wilson, Leach, Henson, and Jones, 1986). For example, microorganisms in a well-oxygenated groundwater containing 4 mg/l of molecular oxygen can degrade only 2 mg/l benzene. Dissolved oxygen should be maintained above the critical concentration for the promotion of aerobic activity, which ranges from 0.2 to 2.0 mg/l, with the most common being 0.5 mg/l (Environmental Protection Agency, 1985b). The concentration of the contaminants affects degradation. Concentrated plumes cannot be degraded aerobically until dispersion or other processes dilute the plume with oxygenated water (Wilson, Leach, Henson, and Jones, 1986).

Many methods have been developed for aerating fluid and semisolid systems above ground (Texas Research Institute, 1982). These include pumps, propellers, stirrers, spargers, sprayers, and cascades--all designed to bring more of the oxygen-deficient material in contact with oxygen in the air. These are discussed in Appendix A, Section 5.2.2.2. Diffusers that sparge compressed air into the groundwater cannot exceed the solubility of oxygen in water, 8 to 10 ppm. Use of pure oxygen can increase the dissolved oxygen content to 40 to 50 ppm, but pure oxygen is expensive and the supersaturated oxygen is likely to degas before the microbes can utilize it (Brown, Norris, and Raymond, 1984). Oxygen levels required for biodegradation and methods for attaining these levels are presented in Appendix A, Section 5.2.2.2.

Alternative sources of oxygen are being considered for increasing levels of dissolved oxygen in a biodegradative system. Hydrogen peroxide, which decomposes to form water and oxygen, can supply much greater oxygen levels (Westlake, Robson, Phillippe, and Cook, 1974). However, concentrations of hydrogen peroxide as low as 200 ppm may be toxic to microbes and levels above 100 ppm may degas to form air bubbles that block the subsurface formations. Ozone can also be used as a source of oxygen, but with the same limitation as hydrogen peroxide in that it is toxic to bacteria and may generate gas bubbles that block the pores in the formation (Lee and Ward, 1984). Use of ozone and hydrogen peroxide for increasing oxygen levels in biodegradation and methods of overcoming their limitations are discussed in depth in Appendix A, Sections 5.1.2.4 and 5.2.2.2.

Appendix A, Section 5.2.2.2, also further discusses the importance of supplemental oxygen for biodegradation in groundwaters and describes possible sources of oxygen and methods of introducing oxygen into contaminated sites.

5.2.2.3 Nutrients

Most groundwater supplies contain a microbial population capable of oil biodegradation (Jamison, Raymond, and Hudson, 1975; Jamison, Raymond, and Hudson, 1976). However, conditions for growth and metabolism of oil may be limited in oxygen, nitrogen, sulfur, phosphorus, and certain trace minerals, although it has been suggested that the levels of most trace elements in aquifers are usually sufficient to support microbial growth (Yang and Bye, 1979). If nutrients are added to the subsurface, there can be a problem with well plugging, from excessive growth at the nutrient release point. An example of this occurred when diammonium phosphate was added to supply nitrogen and phosphorus, which caused excessive precipitation and the procedure had to be discontinued (Raymond, Jamison, and Hudson, 1976).

5.2.2.4 Oxygen-reduction Potential

Degradation of high concentrations of organic compounds may lead to a rapid depletion of the dissolved molecular oxygen and to an eventual decrease in the redox potential in an aquifer (Zeyer, Kuhn, and Schwarzenback, 1986). Aerobic microorganisms that predominate at high redox potentials are replaced by denitrifying, sulfate-reducing, or even methanogenic populations. The redox potential also largely determines the metabolic diversity of the microorganisms in the aquifer.

Many contaminated aquifers will be anaerobic if the microbial population is capable of degrading the material, and it may be possible to use anaerobic *in situ* techniques to treat some compounds (Lee and Ward, 1986), although hydrocarbons degrade slowly under anaerobic conditions. The redox conditions can be controlled to achieve conditions under which specific compounds can be degraded, dehalogenation can be promoted, and particular organisms or enzyme systems can be selected (Appendix A, Section 5.2.2.4).

5.2.3 Alteration of Organic Contaminants

5.2.3.1 Addition of Surfactants

Microbial activity in contaminated aquifers can be enhanced by altering the contaminant chemically or physically to make it more degradable (Ward and Lee, 1984). The dispersal of hydrocarbons in an aqueous system renders them more susceptible to enzymatic attack (Texas Research Institute, 1982). One way to do that is to increase the contaminant surface area and mobility by adding surfactants.

Use of a surfactant can be helpful, but it can also be a mixed blessing (Texas Research Institute, 1982). Since a surfactant will increase the concentrations of both aliphatic and aromatic fractions in the water, the toxicity of the water could possibly increase since the aliphatic molecules may be preferentially degraded. The surfactant itself may also be toxic. The *n*-alkylpyridinium bromides were found to be more toxic than *n*-alkylcarboxylates of identical chain length, confirming that the head group of the amphiphiles plays an important role in the microbial toxicity of surfactants (Beaubien, Keita, and Jolicoeur, 1987). The *n*-alkylcarboxylates appear to be rapidly metabolized by microorganisms in a mixed culture, at least for monologs lower than C_{10}. Cellular toxicity is also dependent upon surfactant hydrophobicity.

See Appendix A, Section 5.1.3.1, for more information on the use of surfactants.

5.2.3.2 Photolysis

Photochemical reactions may be used for the enhancement of compound biodegradation at hazardous waste sites (Sims and Bass, 1984). Photolysis reactions are oxidative and should aid microbial degradation through the oxidation of resistant complex structures (Sims and Overcash, 1981; Crosby, 1971). Such reactions are limited to the surface of the soil or surface treatment of groundwater, but when coupled with soil mixing, may prove to be effective for treating relatively immobile chemicals.

Photolysis can be due either to direct light absorbed by the substrate molecule (direct) or to reactions mitigated by an energy-transferring sensitizer molecule (sensitized photooxidation) (Sims and Bass, 1984). Sensitized reactions result in substrate molecule oxidation rather than substrate isomerism, dehalogenation, or dissociation characteristic of direct photolysis. The reaction rates and breakdown products are only crudely understood. Photolysis can be affected by soil characteristics, such as soil organic content (Spencer, Adam, Shoup, and Spear, 1980), transition metal content (Nilles and Zabik, 1975), soil pigment content (Burkhard and Guth, 1979), and the soil moisture content (Burkhard and Guth, 1979).

Of all atmospheric removal mechanisms, including physical, chemical, and photochemical, the photochemical reactions are the most significant for most classes of hazardous compounds and should be investigated further as a viable treatment option (Cupitt, 1980).

Appendix A describes how to enhance photodegradation and how to assess the potential for use of photodegradation for specific organic compounds.

5.3 OPTIMIZATION OF FRESHWATER, ESTAURINE, AND MARINE BIODEGRADATION

Some of the information in this section may duplicate material covered in Section 5.1, Optimization of Soil Biodegradation, and Section 5.2, Optimization of Groundwater Biodegradation; however, it is presented here under a separate heading, with other related information, to accommodate those readers who may specifically wish to address treatment of freshwater, estaurine, and marine contamination only.

There are considerable differences in the environmental and nutritional parameters, as well as in the microbial communities of aquatic and soil environments (Bartha and Atlas, 1977). The physical behavior of oil pollutants in the two environments is also quite distinct. In surface aquatic environments, horizontal spreading is very rapid and extremely hard to control, while vertical movement is initially restricted. Consequently, the bulk of an oil spill in a surface water body is subject to extensive weathering changes that influence its composition and ultimate fate. A quantitative recovery of an oil slick is almost never feasible, and for some or most of the spill, microbial degradation remains the principal mechanism of removal. However, the degradation rate can be vastly improved by accentuating microbial processes or by adding preacclimated hydrocarbon-degrading microorganisms.

Chronic inputs allow for adaptation of the biological community under the stress of polluting petroleum hydrocarbons, which is different from the stress of sudden inputs occurring from accidental spills (Bartha and Atlas, 1977). Sudden catastrophic spillages often occur in near-shore regions, involving relatively large amounts of contaminant in a small region, and affecting both marine and coastal ecosystems.

The limitations, side effects, and high expense of traditional cleanup techniques have stimulated interest in unconventional alternatives (Bartha and Atlas, 1977).

5.3.1 <u>Biological Enhancement</u>

5.3.1.1 <u>Seeding of Microorganisms</u>

Removal of stranded oil from beach sand or intertidal areas is a use for which seeding for accelerated biodegradation has been considered (Guard and Cobet, 1972). Microorganisms seeded into intertidal areas would have to survive alternate periods of desiccation and exposure to high concentrations of sodium chloride. However, the weathered and partially biodegraded oil on beaches leaves an oil or tar composed of few easily biodegradable components. Since there is a limited oxygen exchange in a sand column, the rates of biodegradation here would be slow.

It is possible to inoculate oil slicks with highly efficient hydrocarbon degraders (Bartha and Atlas, 1977). Some commercial mixtures of microorganisms have been marketed for use in degrading oil in lagoons and other situations (Atlas, 1977). The applicability of seeding selected bacteria and fungi to oil spills has been patented by Azarowicz and Bioteknika International, Inc. (Azarowicz, 1973). However, the full claims of the effectiveness of such organisms remain to be proved. Organisms used for seeding and the conflicting results obtained with this approach are described in Appendix A.

5.3.1.2 Acclimation

Adaptation capabilities of a microbial community are highly variable depending upon site and other factors (Spain and Van Veld, 1983). Adaptation of aquatic microbial communities can last for several weeks after exposure to a xenobiotic compound.

5.3.2 Optimization of Aquatic Factors

5.3.2.1 Temperature

Temperature is a major factor regulating biodegradation of hydrocarbons in seawater (Mulkins-Phillips and Stewart, 1974b). Hydrocarbon degradation is limited in the winter, except at sites previously contaminated and chronically receiving inputs of hydrocarbons and nutrients. Hydrocarbon biodegradation can occur at the low temperatures (<5°C) that characterize most of the ecosystems likely to be contaminated by oil spills (Atlas, 1981). Ninety percent of the oceanic water mass has a temperature of 4°C or below (Morita, 1966). However, the temperature of the water should be 10°C or above to maintain near-maximum bacterial heterotrophic potential.

Low temperatures tend to decrease rates of metabolic oil degradation (Atlas, 1977). Other conditions being equal, moderate to high water temperatures favor the biodegradation approach, while very cold water temperatures would generally contraindicate the use of stimulated biodegradation (Bartha and Atlas, 1977).

In practical terms, little can be done about the temperature of the ocean water (Bartha and Atlas, 1977). In large, open environments, such as oceans, it is not feasible to alter temperatures in an effort to stimulate biodegradation (Atlas, 1977). However, understanding the effects of temperature can allow for optimum conditions for biodegradation to occur.

Seasonal and climatic variability, the types of hydrocarbons involved, and the nature of the site are also important (Colwell and Walker, 1977). In colder climates, there appears to be a seasonal shift to a microbial community capable of low-temperature hydrocarbon degradation (Atlas and Bartha, 1973c). For instance, a higher number of hydrocarbon utilizers capable of growth at 5°C were present in Raritan Bay, NJ, during winter than during other seasons. In this situation, rates of hydrocarbon mineralization measured at 5°C were significantly higher in water samples collected in winter than in summer.

Temperature effects depend upon the length of time involved (Nedwell and Floodgate, 1971). The short term reflects the response of a given population to the change of temperature, while the long term may see the development of a different population that is adapted to the new conditions. The temperature optima for the heterotrophic utilization of glucose and amino acids in lake sediments decrease with decreasing ambient temperature, suggesting adaptation by or selection for psychrotrophic populations (Bartholomew and Pfaender, 1983). Although microbes in colder waters are adapted to lower temperatures, biodegradation rates can be expected to be much slower, and bioreclamation will take longer in the extreme North. Moderate degradation rates have been observed even at Arctic temperatures (Traxler, 1972), but the rates may not be adequate to rapidly remove hydrocarbon contaminants (Atlas, 1981). Seed

microorganisms for such environments will have to be psychrophilic or psychrotrophic, if they they are to be capable of growth and active metabolic degradation of oil (Atlas and Bartha, 1972a).

The effects of temperature differ, depending upon the hydrocarbon composition of a petroleum mixture (Atlas and Bartha, 1972a) (see Appendix A, Section 5.3.2.1). Low temperatures retard the rates of volatilization of low-molecular weight hydrocarbons, some of which are toxic at low concentrations to microorganisms. The presence of such toxic components has been found to delay the onset of oil biodegradation at low temperatures. There is some preference for paraffin degradation, especially at low temperatures. At low temperatures, cometabolism appears to play an important role in determining the rates of disappearance of hydrocarbons (Horowitz and Atlas, 1977a).

Temperate zone lakes have, in general, more extreme water temperature variations than oceans (Bartha and Atlas, 1977). In temperate lakes of Wisconsin, water temperature was the predominant limiting factor throughout the fall, winter, and spring, and only during the summer did nutrient limitation take precedence (Ward and Brock, 1976).

5.3.2.2 Oxygen Supply

Aquatic environments with low concentrations of dissolved oxygen may have lower degradation potentials. Limitation by oxygen deficiency is least likely to occur in cases of thin, floating oil slicks, but the interior of floating water-in-oil emulsions (chocolate mousse) and tar globules of substantial size may well become anaerobic (Bartha and Atlas, 1977).

At pH values between 5 and 8, mineralization of hydrocarbons in estuarine sediments is highly dependent upon oxygen availability (Hambrick, DeLaune, and Patrick, 1980). Rates of hydrocarbon degradation decrease with decreasing oxygen reduction potential. Therefore, hydrocarbons would persist in reduced sediments for longer periods of time than would hydrocarbon contaminants in aerated surface layers. Low mineralization (about 10 to 20 percent) of some alkanes, but not of naphthalene or hexadecane, occurred under anaerobic conditions. Oxygen was not limiting in the water column of three lakes tested; however, it most probably was a limiting factor for utilization of hydrocarbons in the lake sediments, where most of the hydrocarbons were concentrated. Sinking the oil into anoxic sediments is an undesirable treatment method. Many hydrocarbons that enter anaerobic environments, such as reduced sediments, could persist there indefinitely as environmental contaminants (Atlas, 1981).

Forced aeration can be used to supply oxygen in lagoon and many freshwater environments (Rosenberg, Englander, Horowitz, and Gutnick, 1975). Oil biodegradation in the water column of deep lakes is often oxygen limited at various times of the year (Ward and Brock, 1974). Oxygen is rarely a limiting factor in the decomposition of oil in the open ocean, and the rate of oxygen uptake is linear to the rate of nitrogen uptake (Floodgate, 1976). It would generally be impractical to use forced aeration here (Ward and Brock, 1974). Marine oil spills must generally rely on wave action to achieve the necessary aeration. Oxygen might be supplied by employing algae in open, as well as contained, systems (McLean, 1971). Besides supplying oxygen to the water column, some algae have been found to be capable of heterotrophic metabolism of hydrocarbons (Walker, Colwell, and Petrakis, 1975).

The theoretical oxygen demand is 3.5 g of oil oxidized per g of oxygen (Floodgate, 1979; ZoBell, 1969). The dissolved oxygen in 3.2 x 10^5 l of seawater would, therefore, be required for the complete oxidation of 1 l of oil (ZoBell, 1969).

5.3.2.3 Nutrients

Although temperature is the main limiting factor much of the year, nutrient deficiencies can limit oil biodegradation in temperate lakes, during the summer (Ward and Brock, 1976). One of the most important factors that limit the rate of decomposition of oil in the marine environment is the concentration and rate of supply of inorganic nutrients, especially nitrogen and, to a lesser extent, phosphorus (Atlas and Bartha, 1972b). Marine microbial populations are also limited by the levels of organic matter present (Atlas, 1977).

In marine environments where available phosphorus and nitrogen are plentiful, oxidation of oil might be relatively rapid (Floodgate, 1976). Whether nitrogen and phosphorus are going to be a limiting factor in seawater may depend upon whether the contaminant is an oil slick or composed of soluble hydrocarbons (Atlas, 1981). In an oil slick, nitrogen and phosphorus must be able to diffuse to the slick, and rates of diffusion may be inadequate to supply sufficient nutrients to establish optimal C/N and C/P ratios for microbial growth and metabolism. With soluble hydrocarbons, on the other hand, nitrogen and phosphorus are probably not limiting, since the solubility of the hydrocarbons is so low as to preclude establishment of an unfavorable C/N or C/P ratio.

The oxidative state of the inorganic nitrogen is immaterial; both nitrate and ammonia work well (Floodgate, 1976). An oleophilic nitrogen and phosphorus fertilizer is described in Appendix A, Section 5.3.2.3. Some of the nitrogen becomes part of the bacterial biomass and is incorporated into the cell structure, while some forms enzymic molecules needed for oil decomposition (Floodgate, 1976). The amount of nitrogen ("nitrogen demand") required for degradation of a given amount of oil contaminant can be calculated (see Appendix A, Section 5.3.2.3).

Beaches tend to concentrate the above elements to about three times the level found in seawater (Floodgate, 1976). The inorganic nutrients may be less of a limiting factor for oil biodegradation on beaches, since each tide may bring a new supply. An important factor appears to be the way the oil arrives and its degree of division; e.g., lumps are more recalcitrant than a thin skim (Davis and Gibbs, 1975). On a high energy beach, much of the oil will be washed away by wave action (Floodgate, 1976). Otherwise, it can become buried and the conditions anaerobic, with a slowing of the digestion. Ironically, a limiting factor of oil degradation on beaches may be the lack of water for microbial decomposition of the material (Atlas, 1981).

An estuarine area has a much higher bacterial count than offshore areas, possibly because of the availability of more nutrients. Eutrophic lakes and ponds appear to be better at degrading organic compounds than oligotrophic systems.

The need for mineral nutrients is generally recognized, but the addition of nutrients in the form of water-soluble salts restricts the practical application of stimulated oil biodegradation to oily water contained in tanks, bilges, or holding ponds (Rosenberg, Englander, Horowitz, and Gutnick, 1975). In enclosed systems, addition of mineral-containing water will supply many of the required nutrients (Atlas, 1977). Supplemental nitrogen and phosphorus would still have to be added for extensive oil biodegradation. These nutrients could be added in a water-soluble form, such as ammonium phosphate in these situations (Rosenberg, Englander, Horowitz, and Gutnick, 1975).

Treatment of free-floating oil slicks requires oil- rather than water-soluble nitrogen and phosphorus supplements in a form that will not allow it to dissipate from the oil-water interface, i.e., oleophilic fertilizers. These fertilizers consist of paraffinized urea and octyl phosphate and are selectively available to oil-degrading microorganisms only (Atlas and Bartha, 1973a). Application of oleophilic fertilizers also prevents algal blooms, which would follow the use of water-soluble formulations.

5.3.3 Alteration of Organic Contaminants

The physical state of petroleum hydrocarbons has a marked effect on their biodegradation (Atlas, 1981). Most petroleum hydrocarbons are soluble in water at very low concentrations, but most oil spill incidents release petroleum hydrocarbons in concentrations far in excess of the solubility limits (Harrison, Winnik, Kwong, and Mackay, 1975). The degree of spreading determines, in part, the surface area of oil available for microbial colonization; in aquatic systems, the oil normally spreads, forming a thin slick (Berridge, Dean, Fallows, and Fish, 1968). The degree of spreading is reduced at low temperatures because of the viscosity of the oil.

Petroleum that is discharged into the sea is acted upon by a variety of processes that cause changes in its physical and chemical characteristics (Tjessem and Aaberg, 1983). Spreading on the surface, followed by evaporation and emulsification appear to be the dominant initial processes (368) (see Appendix A, Section 5.3.3). These processes are highly dependent upon temperature, wind, and sea waves, as well as the exact chemical composition of the particular oil (Payne and Jordan, 1980).

5.3.3.1 Addition of Surfactants

In a two-phase liquid medium where the bulk of the carbon and energy source is water insoluble and all other mineral nutrients are dissolved in the water phase, microbial growth typically occurs at the interface of the two liquids (Bartha and Atlas, 1977). The interfacial tension reduces this interface to a minimal area. Microorganisms can lower the interfacial tension, thereby, increasing the interface and the accessibility of the hydrocarbon substrate. This phenomenon is apparently due to incidental leakage of fatty acids and other metabolic intermediates. In fact, fatty acids are common emulsifying agents. Many oil-degrading microorganisms produce emulsifying agents (Reisfeld, Rosenberg, and Gutnick, 1972), and there is interest in the use of microbes as oil dispersants, since they would have low toxicity for cleanup of oil and cause minimal environmental damage.

In aquatic ecosystems, oil tends to spread naturally, approaching a monolayer in thickness (Fay, 1969). A greater surface area in these ecosystems can also be achieved by emulsification (Berridge, Dean, Fallows, and Fish, 1968). Use of emulsifiers on a slick to increase the spreading of the oil and its exposure to sunlight (causing photolysis) may help increase the rate of degradation (Tissot and Welte, 1978) (see Appendix A, Section 5.3.3.1). If there are no adverse toxic effects, dispersion of oil should also accelerate microbial hydrocarbon degradation (Atlas, 1981). This is an important consideration when deciding whether dispersants should be added to oil spills. However, some dispersants may contain chemicals that are inhibitory to microorganisms, and increased toxicity must also be considered in their use.

It has been found that addition of oil to an aqueous mixed culture system with mild agitation forms an oil-in-water emulsion within 24 hr (Zajic and Supplisson, 1972). On continued exposure this reverts to a water-in-oil emulsion in 48 hr. Functional groups in the oil that are hydrophilic and contribute to these reactions are: --COOH (carboxylic), --OH (alcohol), --CHO (aldehyde), --OSO_3 (sulfate), and --SO_3H (sulfonate). Normally, the water-in-oil emulsion is associated with petroleum having asphaltenes. The variation in size of the encapsulated water droplets appears to correlate with the stability of the emulsion. This emulsion is very stable with a droplet size of an estimated average diameter of 0.5 u. Microbes are able to exist in the water capsules within the emulsion, where they selectively remove paraffinic hydrocarbons and low molecular weight aromatics.

Appendix A, Section 5.3.3.1, describes some efficient microbial emulsifiers and the result of emulsification of oil spilled on beaches.

5.4 TREATMENT TRAINS WITH CHEMICAL AND BIOLOGICAL PROCESSES

Contaminated water and wastewaters will normally be composed of a complex mixture of compounds varying in concentration (Sutton, 1987; Wilson, Leach, Henson, and Jones, 1986). The compounds may be degradable, inhibitory, or recalcitrant to various degrees. In most contaminated hydrogeologic systems, a remediation process is so complex in terms of contaminant behavior and site characteristics that no one system or unit will usually meet all requirements. Often it is necessary to combine several unit operations, in series or parallel, into one treatment process train to restore groundwater quality to an acceptable level.

Physical-chemical treatment techniques may be required to render the water or wastewater less inhibitory to microbial treatment or to ensure the removal of nonbiodegradable compounds. Physical-chemical treatment will normally be provided in conjunction with the biological step (Wilson, Leach, Henson, and Jones, 1986). Barriers and hydrodynamic controls serve as temporary plume control measures. However, hydrodynamic processes must also be integral parts of any withdrawal and treatment or <u>in situ</u> treatment measures. Heavily contaminated soils may have to be removed or attenuated (Brubaker and O'Neill, 1982). Then, pumping systems can be installed to remove free product floating on the groundwater before biorestoration enhancement measures are initiated to degrade the more diluted portions of the plume (Wilson, Leach, Henson, and Jones, 1986). A limiting factor is moving the contaminated subsurface material to the treatment unit, or getting the treatment process to the contaminated material, in the case of <u>in situ</u> processes. Withdrawal and biological treatment seem to currently be the most effective methods for biological restoration of aquifers contaminated by organic compounds (Knox, Canter, Kincannon, Stover, and Ward, 1968).

Physical processes, implemented quickly and efficiently, can retrieve large quantities of the contaminant (Dietz, 1980). Salvage wells for oil average 30 percent recovery. Pumping of drilled wells recovered two-thirds of a gasoline spill in one study (Raymond, Jamison, and Hudson, 19761). Biodegradation has sometimes been applied as a treatment for spill management after an unsuccessful attempt to recover all of a contaminant by physical means; i.e., pumping, air stripping, or vapor extraction (Raymond, Jamison, and Hudson, 1976; Walton and Dobbs, 1980). These auxiliary physical treatments can also be employed during bioreclamation (Brubaker and O'Neill, 1982). Integration of these removal mechanisms into the biological step will represent a cost-effective alternative, if technically feasible. Biodegradation is an alternative to physical recovery processes once they become nonproductive in terms of cost and effectiveness (Buckingham, 1981). One case study began stimulating soil organisms after estimating that physical recovery methods would require 100 years of operation and maintenance to make the contaminated water potable (Raymond, Jamison, and Hudson, 1976).

A thorough understanding of the hydrogeologic and geochemical characteristics of the area will permit full optimization of all possible remedial actions, maximum predictability of remediation effectiveness, minimum remediation costs, and more reliable cost estimates (Wilson, Leach, Henson, and Jones, 1986). Treatment techniques that can be used in combination with bioreclamation to reduce toxic concentrations to tolerable levels are discussed in Appendix A, Section 5.4.

5.5 BIODEGRADATION IMPLEMENTATION PLAN

Before a biodegradation program is begun, laboratory and field investigations would have to support the hypothesis that a significant number of the toxicants present in the soil could be degraded by natural processes (Buckingham, 1981; Sommers, Gilmore, Wildung, and Beck, 1981; Arthur D. Little, Inc., 1976; Quince and Gardner, 1982). The biodegradability of the contaminants must be assessed; the source, quantity, and nature of the spilled material must be determined; and the environmental conditions of the site must be considered.

A qualitative and quantitative analysis of the contaminants present would have to be made. The rates and products of degradation of each contaminant, as a function of manipulable soil parameters, would need to be established. The rates and products of degradation of mixtures of contaminants, as a function of soil parameters, would also have to be known. The geology and hydrogeology of the site and the extent of contamination must be assessed, including formation porosity, hydraulic gradient, depth to water, permeability, groundwater velocity, and direction and recharge/discharge quantification. A laboratory investigation on the potential for inhibition of the microorganisms, their oxygen and nutrient requirements, and the effects of temperature is necessary. Finally, the extent of degradation achieved under field conditions must be determined.

The optimum conditions would then be established for each of the contaminants. There would have to be an integration of each set of optimum conditions into an overall plan of action, considering sequencing of treatment processes and the particular toxicants found together in given areas. The quantity and character of the contaminants and their location in the aquifer will determine what containment technique, recovery method, and treatment system(s) should be used. The environmental conditions would be manipulated according to the plan of action, and the progress of toxicant degradation would be monitored.

Detailed procedures for a biodegradation implementation plan are presented in Appendix A, Section 5.5.

SECTION 6

POTENTIAL LIMITATIONS OF BIODEGRADATION

In situ bioreclamation is a versatile tool for treating contaminated groundwater; however, it is not the answer to all contamination problems (Brown, Loper, and McGarvey, 1986). Its applicability must be determined for each site and depends upon local site microbiology, hydrogeology, and chemistry.

There are several important limitations for application of biological treatment (Niaki, Pollock, Medlin, Shealy, and Broscious, Draft). These include: 1) environmental parameters must be appropriate for support of microbial growth (pH, temperature, redox state, and nutrients); 2) some chemicals are nonbiodegradable, according to current knowledge; 3) by-products of biodegradation may be more toxic or persistent than the original compound (e.g., DDT-DDD); 4) substrate concentration may be too high (toxic) or too low (inadequate energy source); and 5) complex mixtures of organics may include inhibitory compounds.

Bioreclamation does not elicit destruction of heavy metals and some recalcitrant organics (Lee, Wilson, and Ward, 1987), although microorganisms can promote change in chemical form and mobility of hazardous materials. Introduction of nutrients and the residues generated by the organisms may adversely affect water quality. The process may not work well for aquifers with low permeabilities that prevent adequate circulation of nutrients and dissolved oxygen. A field demonstration using hydrogen peroxide as a source of oxygen in a very gravely clay loam was not very successful, due partly to the low permeability (1×10^{-6} cm/sec), which made it difficult to inject nutrients and transport oxygenated water (Science Applications International Corporation, 1985a). Other factors contributing to the poor success of this demonstration were the complexities of the site, possible mobilization of lead and antimony by the hydrogen peroxide treatment, and reductions in the permeability of the soil due to precipitation of the nutrients.

Some partial degradation products might be more toxic than the parent compounds (Lee, Wilson, and Ward, 1987). Transformation of a toxic organic solute is no assurance that it has been converted to harmless or even less hazardous products (Mackay, Roberts, and Cherry, 1985). For example, anaerobic biotransformation of common groundwater contaminants, such as PCE and TCE, can result in the formation of intermediates, such as vinyl chloride, which cannot be further transformed under prevailing reducing conditions. Given our limited understanding of transformation processes and the factors influencing them, hazardous contaminants must be assumed, in the absence of site-specific evidence to the contrary, to persist indefinitely.

Bacterial growth can plug the soil and reduce groundwater circulation (Lee, Wilson, and Ward, 1987). The plugging of well screens and the neighboring interstitial zones of the aquifer can be a direct result of biofilm generation (Cullimore, 1983). This can result in reduced flow from the wells, sometimes causing a complete shutdown of the system. Such water supply wells can reduce water quality (through the generation of turbidity, taste, odor, and

color) and, eventually, lead to the generation of serious anaerobic corrosion problems. Degeneration in well productivity has, on occasion, been expensive, with an estimated annual cost at between $10 million and $12 million (Canadian). Organisms found to be responsible for this plugging have been Gallionella, other bacteria able to deposit iron or manganese oxides or hydroxides in or around the cell (e.g., Leptothrix, Crenothrix, or Sphaerotilus), and heterotrophic bacteria able to grow in a biofilm. The extensive growth of organisms at the aquifer/well interface is probably due to the increase in oxygen concentration at the site of injection. However, disinfectants and physical techniques have been reported for controlling this problem.

Microorganisms can mobilize hydrocarbons by transforming them to polar compounds, such as alcohols, ketones, and phenols, or to organic acids, such as formate, acetate, proprionate, and benzoate, such as when a site is contaminated with JP-5 (Perry, 1979; Ehrlich, Schroeder, and Martin, 1985).

It appears that microorganisms that are able to degrade chemicals in culture sometimes may not do so when introduced into natural environments because of improper pH, inability to survive, or use of other substrates (Zaidi, Stucki, and Alexander, 1986). Some Pseudomonas strains were able to mineralize biphenyl or p-nitrophenol in lake water at the natural pH of 8.0, while another strain required the pH to be adjusted to 7.0, and yet another did not mineralize the substrate although its population density rose.

To be a useful pollution abatement method, biodegradation of petroleum pollutants would have to occur rapidly enough to prevent contamination of groundwater or movement of pollutants off site (Atlas, 1977). This may not always be the case. It is likely that the treatment could cost in the range of tens of thousands of dollars for simple treatment programs up to tens of millions of dollars for complex, large sites (Lee, Wilson, and Ward, 1987). This, however, compares favorably with costs of hundreds of millions of dollars that could be incurred by using other methods.

Because of the uncertainty involved in applying innovative remedial approaches, there will always be reluctance to test their feasibility (Alexander, 1980). Uncertainty about subsurface geochemical and contamination conditions makes it difficult to provide specific effluent and other data typically required in a permitting process. The complexity of the technologies and the permitting process itself will cause delays that can hinder cost-effective feasibility demonstrations.

There are few additional safety hazards associated with in situ bioreclamation aside from those hazards normally associated with being on a hazardous waste site or a drill site (Environmental Protection Agency, 1985b). Since wastes are treated in the ground, the danger of exposure to contaminants is minimal during a bioreclamation operation relative to excavation and removal.

SECTION 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

1. This country is faced with an enormous task of cleaning up hazardous materials accidently or indiscriminately introduced into the environment. Many of the standard techniques employed for treating the contaminated soil and groundwater have not been totally effective or have not offered a permanant solution to the problem. Some have even created additional hazardous sites.

2. Various biological techniques have been applied to treating this contamination. Among these, in situ biodegradation is receiving growing support and widespread testing in the field. In situ biodegradation practices can be combined with on-site biodegradation techniques, as required, to offer a very efficient and cost-effective treatment system.

3. In situ biodegradation techniques can also be employed in conjunction with chemical and physical procedures, as warranted by a given contamination incident. Treatment trains that combine biodegradation with physical and/or chemical remediation methods should optimize conditions for the microbes to facilitate total destruction of the contaminant materials.

4. In most episodes of soil and aquifer contamination, indigenous microorganisms present at the site appear to be able to degrade the material, if sufficient nutrients and oxygen are supplied and other environmental factors modified, as necessary.

5. In situ biodegradation is still a developing technology. A great deal of effort is being directed toward laboratory investigations of hydrocarbon-degrading microorganisms and their requirements for optimum degradation. Metabolic pathways are being elucidated to be able to predict the performance of individual strains and mixtures of organisms. New microbes are being developed to target a wider range of substrates, as well as the more recalcitrant contaminants. Manipulation of environmental factors is being attempted to improve the conditions for the desired organisms. Anaerobic treatments are also being explored, as well as combination of anaerobic with aerobic measures.

6. In situ biodegradation is a very complex process. The interactions of many factors have yet to be understood before a program of specific measures can be applied with certainty in the field to be able to predict and direct the outcome of the treatment. Although this may appear to be an overwhelming task, much useful information has already been obtained, and research is progressing in promising directions. When there is sufficient evidence that this approach (or its combination with on-site, physical, or chemical procedures) is faster and more economical than other methods, and contaminated sites continue to be discovered, the funding may become available from both Government and industry to encourage full development of the technology.

7. Genetic engineering of microorganisms may be able to help concentrate and

improve performance of their hydrocarbonoclastic capabilities. Developments in this area will depend upon an increased understanding of microbial physiology, the basic biochemical and genetic basis for microbial detoxifications, and an understanding of microbial resistances to antibiotics and heavy metals. The organisms must also have a competitive advantage over the indigenous organisms to be able to survive long enough to accomplish their task. Therefore, genetic enhancement of ecologically important traits may be required.

8. Development of specialty organisms with unique capabilities and the use of fungi, algae, and actinomycetes are being explored for biodegradation applications. However, the value of seeding contaminated sites with supplemented microorganisms has yet to be substantiated.

9. There is still much to be learned about *in situ* biodegradation processes. Laboratory results will have to be verified in the field. Unfortunately, few field applications have been conducted as controlled experiments. Inappropriate or inadequate measurements are often taken or too many variables are introduced, including the use of other restoration measures concurrently with the enhancement techniques. Indirect evidence is sometimes offered as proof that the procedure is responsible for the observed reduction in contaminant levels. More emphasis should be placed on devising methods that will furnish conclusive evidence that the process, or the particular variable being tested, can be linked with disappearance of the contaminant.

7.2 RECOMMENDATIONS

The underlying limitation to using technologies associated with in situ treatment of organic deposits is that these methods are often in an experimental stage of development. This finding frustrates efforts to systematize selection of reagent and delivery and recovery systems, and it emphasizes the need for laboratory simulation and testing prior to implementation. Each site at which in situ treatment is considered becomes, in essence, a research project, but the use of in situ methods on a large scale is hampered by the lack of field experience. There is little incentive for site managers to consider their use.

It became apparent through review of the literature that there are significant gaps in the understanding of the factors involved in the use of bioreclamation for treatment of contaminated soils and groundwaters. The following are suggestions for areas where additional research could contribute useful information toward development of a more effective technology.

7.2.1 Microorganisms

1. The methods for sampling and characterizing indigenous subsurface microorganisms should be improved. This should include methods for assessing the degradative capabilities of the organisms present. Methods for detection, identification, enumeration, and biochemical activity of introduced organisms can help monitor their survival and functioning in the field.

2. Since a variety of methods are being used to investigate in situ biodegradation, such as techniques for microbial enumeration, comparison of results from different studies can be difficult. Efforts should be made to standardize methods where possible and feasible, even as new and better approaches are developed.

3. Nitrogen-fixing and oxygen-producing hydrocarbonoclastic microorganisms should be sought for possible application to nutrient-deficient environments.

4. In many cases, wastes contain carbon sources that microorganisms may use selectively as nutrient sources instead of the targeted toxic chemical. Development of, or selection for, organisms that can utilize the toxic fractions is necessary.

5. Studies are needed on the population dynamics and survival of new and existing organisms in the environment to permit continuing and long-term biodegradation. This should include requirements of the organisms for growth under field conditions.

6. Before a microorganism can be tested outside a contained environment, determination of its fate after it has done its work is needed. Predictive ecological research is essential to ensure that biodegradation of oily wastes do not unintentionally create new and potentially serious environmental problems.

7. Research should be conducted into the need of microorganisms for acclimation to the compounds they will eventually degrade and, ultimately, lead to shortening the acclimation period.

8. Microorganisms with special capabilities, such as the ability to metabolize contaminants at low temperatures, resistance to high concentrations of pollutants, the ability to adhere to solid surfaces or nonaqueous-phase liquids, resistance to heavy metals, the ability to produce emulsifiers, the capacity to function at high salt concentrations, or are "superbugs," should be sought.

9. While aerobic bacteria and fungi may prove to be the most useful microbes for biodegradation, other organisms, such as anaerobes, algae, and actinomycetes should also be investigated.

7.2.1.1 Seeding

1. The value of seeding contaminated sites with microorganisms has yet to be substantiated. While seeding soil with an acclimated or mutant microbial population to remove contaminants holds a great deal of promise, especially when concerned with recalcitrant substances, results from previous attempts have been inconclusive. Further research is required to determine whether seeding with microbes is an effective and cost-effective technique for the restoration of contaminated aquifers and soils.

2. Methods should be devised to determine whether indigenous or supplemented microorganisms would be preferred in a given contaminant incident.

3. Research is needed to allow prediction of whether introduced organisms require nutrients, surfactants, or other amendments to increase their activity. The appropriate requirements for manipulation of the environment should be established for the intended organisms. Studies should determine the requirements of supplemented organisms to be able to outcompete indigenous microflora.

4. The problem of developing potentially pathogenic microorganisms in the subsurface or long-term environmental effects of seed cultures requires further investigation. Many of the hydrocarbonoclastic microorganisms are of the same genera that contain potentially pathogenic species. For example, many hydrocarbon-degrading bacteria are Pseudomonas species. Establishing that seed microorganisms do not cause a public health threat will be difficult because it must be determined what organism to use in the toxicity testing. This would also require bioassay testing for the different environments into which seeding is considered. The direct toxicity of petroleum and oily wastes must be considered, and it should be determined whether microbial seeding would increase or decrease the undesirable effects of oil pollutants. A consideration of toxicity requires studies on sublethal, as well as lethal effects.

5. The reasons for the frequent failure of introduced microorganisms to survive and degrade contaminants should be determined and overcome.

6. More work should be performed to develop seed inocula consisting of various groups of microorganisms that will interact in a predictable manner to degrade specific mixtures of contaminants.

7. The possibility of seeding with specialty organisms with unique capabilities should be explored for meeting a variety of demands. This could

include microbes that are active in cold climates, are salt tolerant, are resistant to heavy metals, are "superbugs," or are able to produce emulsifiers. Greater use could be made of fungi, algae, and actinomycetes.

7.2.1.2 Genetic Engineering

1. Development of genetically engineered microorganisms suitable for rapid and more complete destruction of specific organic compounds is currently an active area of research, which may lead to advances in both above-ground and *in situ* biological treatment technologies. More information is needed on the ability of genetically engineered organisms to survive, grow, and function in the soil environment, as well as any potential permanent negative influences.

2. The genes responsible for biodegradation should be determined to permit cloning and introduction of the genes into desired recipients and to allow regulation of the function of the introduced gene, as required, without negative effects on the recipient or the environment.

3. Additional research to address the controversy of mutant *versus* natural bacterial stimulation should lead to selection of the most time-efficient and cost-beneficial procedures.

4. Since many of the waste pool constituents are mutagens and the degradative traits are often associated with nonchromosomal genetic material (plasmids), the organisms could mutate to ineffective strains. This needs to be considered when developing suitable microbial strains for field use, such as utilizing chromosomal genes or more stable plasmids.

5. Genetically engineered microorganisms should be developed that can destroy pollutants that might otherwise not be destroyed or only at a slow rate. Multiple degradative pathways might be incorporated in a single engineered strain.

6. The environmental constraints for the particular engineered organism should be determined.

7. Engineered organisms might be particularly useful in bioreactors, where the environmental conditions can be better controlled.

7.2.1.3 Biochemistry

1. More research is needed on the metabolism and metabolic pathways for the biodegradation of specific compounds by different microorganisms or groups of microorganisms, including algae, fungi, and oligotrophic bacteria.

2. The importance in the environment of the various pathways for hydrocarbon biodegradation should be determined. Various biological strategies exist for the microbial utilization of petroleum hydrocarbons. The natural environments that receive petrochemical hydrocarbons should be analyzed for intermediate products to determine which pathways are actively used by microbial populations in natural ecosystems. It is likely, but as yet unproven, that different pathways will be active under different conditions, e.g., at different hydrocarbon concentrations.

3. While it has been shown that most PAHs are biodegradable and catabolic pathways for a few have been determined, much more research is needed in this area. Careful attention should be given to the potential carcinogenicity and mutagenicity of intermediate products formed during their biodegradation.

4. Microorganisms may use a water-soluble substrate as it spontaneously dissolves in water, or they may metabolize the compound after a biologically mediated solubilization or by mechanisms involving physical contact with the insoluble phase of the substrate. Former studies have been concerned only with comparisons of dissolution and degradation rates. Additional study is needed to assess the significance of other mechanisms by which microorganisms utilize water-insoluble organic chemicals.

5. The pathways of biodegradation in surface waters, sediments, soils, and subsurface materials should be studied.

6. Research should be conducted into the mechanisms of biodegradation to be able to predict the fate of toxic molecules in the environment and to aid in the design of new and innovative reactor systems that exploit those mechanisms.

7. The kinetics of biodegradation should be investigated, including kinetics of chemicals that are acted on by cometabolism, that are sorbed, and that are present in nonaqueous-phase liquids.

7.2.1.4 <u>Cometabolism</u>

1. Aside from traditional fermentation and growth of microorganisms on hydrocarbons, cooxidation and stationary transformation techniques are promising areas of hydrocarbon biotechnology for the future. These are systems actually operating in nature and need to be more fully explored for application in a biodegradation treatment program.

2. Since soil microorganisms capable of cometabolically degrading a xenobiotic may not proliferate on the xenobiotic substrate without their cometabolic companions, unique techniques may be necessary to isolate microorganisms with this capability. There is limited information available suggesting what alternate substrates would be suitable for isolating microbial populations capable of metabolizing xenobiotics by cometabolic processes. Such techniques might utilize, as a primary substrate, a chemical analog that would permit enrichment of microbial populations having the cometabolic requirements to degrade the xenobiotic.

3. Evidence is accumulating that indicates that cometabolism may be a particularly important phenomenon in the dissipation of the more refractory xenobiotics. However, considerably more research is needed to understand the importance of this process.

7.2.1.5 <u>Enzymes</u>

1. Theoretically, enzymes would quickly transform hazardous compounds if they remained active in the soil, and oily wastes are amenable to treatment by application of cell-free enzymes. The potential level of treatment is high. However, there is little information available on the use of this technique in soil and no information from the field. Its reliability is unknown. It is

even possible that the enzymatic degradation products may not be less hazardous than the parent compounds. All these issues should be investigated to determine whether using enyzmes for treating hazardous contaminants is feasible and, if so, to establish a protocol for their application.

2. Extracellular enzymes capable of degrading xenobiotics or xenobiotic degradation products exist in the soil. The presence of peroxidases in soil should be of great interest to researchers investigating soil xenobiotic transformations. The role of lignin peroxidases in the degradation of polluting chemicals should be further explored. Presumably, these enzymes could be involved in many soil metabolic reactions affecting xenobiotic residues.

7.2.1.6 Interactions

1. Most present knowledge of microbial degradation of different hydrocarbons has been obtained in pure cultures of microorganisms containing single hydrocarbon substrates. Little is known about how natural mixtures of microorganisms degrade each chemical component when they are present in the total petroleum sample.

2. Aged oily wastes usually support large indigenous populations of bacteria, fungi, and actinomycetes. The exact interacting role of these microorganisms in decomposition or the nature of their interactions with the natural soil microflora is unknown. A thorough assessment is needed to establish their potential role in the degradation of chemical constituents.

3. Each bacterial strain will generally degrade only one particular chemical or closely related groups of chemicals. Little research has been conducted on developing protocols for using single species of microorganisms or defined microbial consortia to treat complex waste mixtures.

4. The rates of biodegradation of hydrocarbons from oil spills appear to be highly dependent upon localized environmental conditions. The fate of many components in petroleum, the degradative pathways that are active in the environment, the importance of cooxidation in natural ecosystems, and the role of microorganisms in forming persistent environmental contaminants from hydrocarbons, such as the compounds found in tar balls, are unknown and require future research. Although a number of rate-limiting factors have been elucidated, the interactive nature of microorganisms, oil, and environment needs to be better understood.

7.2.2 Environmental Factors

1. The environmental factors that limit the rate or extent of biodegradation or that prevent successful bioremediation must be established for both indigenous and introduced populations. The limiting factors should be overcome by practical means.

7.2.2.1 Aerobic/Anaerobic Conditions

1. Further biodegradation research is needed in the area of oxygen enhancement to develop more efficient aeration techniques for stimulating aerobic biodegradation in soils and groundwater. Innovative approaches should

be sought, such as provision of electron acceptors other than oxygen or the use of very small gas bubbles to provide oxygen.

2. Microbiological plugging in wells usually occurs through biofilm growth constricting the flow of water entering the well through the screen slots and the interstitial zone in the aquifer immediately surrounding the well, probably as a result of the increase in oxygen concentration there. The mechanism of biofilm generation as the critical component in biological plugging should be studied in the well screen/aquifer environment to find a way to eliminate this problem.

3. There is a lack of data for applying an anaerobic degradation system to treat dilute, low-organic-strength wastewater, such as contaminated groundwater, although it is very promising.

4. There have been a few reports on the anaerobic degradation of some petroleum hydrocarbons in natural ecosystems using nitrate, iron, or sulfate and carbon dioxide as alternate electron acceptors during anaerobic respiration. Nitrate has been reported to support the degradation of xylenes in subsurface material. This approach is still experimental but is believed to offer considerable promise. It should be further studied since nitrate is inexpensive, is very soluble, and is nontoxic to microorganisms (although there are environmental restrictions on it). Degradation of all hydrocarbon groups, however, has not been biochemically confirmed in either pure or mixed cultures, and this needs to be explored. The criteria used for assessing anaerobic hydrocarbon degradation have not been adequate to establish definitive results (e.g., anaerobic biodegradation of benzene has been inferred but not confirmed). Therefore, such criteria must be developed to be able to validate these techniques.

5. The possibility that the biodegradation of polluting oil in nitrogen-deficient natural environments is aided by atmospheric nitrogen fixation needs to be further explored.

7.2.2.2 Temperature

1. The ability to actively metabolize hydrocarbons at low temperatures should be further investigated. Psychrophilic oil-degrading microorganisms should be sought for activity in cold marine and cold soil environments.

2. Composting allows an enrichment of thermophilic microorganisms that have their optimum degradative activity between 50° and 60°C. It may offer considerable potential for maximizing the biodegradation rate of oily wastes. It might also provide for the initial detoxification or degradation of these wastes prior to their application to land treatment systems. Research is needed to fully assess the potential of composting for detoxification, degradation, and inactivation, especially recalcitrant organic constituents.

3. There is minimal literature that documents attempts, successful or unsuccessful, to significantly increase the temperature of the soil in the field. Fairly simple field tests need to be performed to determine whether application of materials, such as placing black PVC sheeting on the soil surface or using it as a mulch, would increase the temperature enough to cause significant increases in degradation rates.

7.2.2.3 Nutrients

1. While there may be a substantial amount of potentially available nutrients in many wastes, their rate of release from organic material may be too slow to sustain microbial activity. Research is needed to determine the rate and extent to which degradation of petroleum products can be increased by addition of available nutrients for microbial utilization.

2. Nutrient injection into groundwater monitoring wells is an area in need of research. It is probable that subsurface injection is economically feasible when compared with methods of surface application. Methods of fertilizer injection into the subsurface needs to be evaluated.

3. Nutrient requirements of indigenous or introduced organisms should be established to optimize their growth and degradation of the pollutants.

4. Methods are needed for introducing the correct amounts of nutrients to locations where they are required and in the form needed.

7.2.2.4 Water Potential

1. Much more information is needed on the effects of water potential on the ability of different microbial species to degrade or detoxify individual components of hydrocarbons. Such information is important in developing management strategies for efficient and effective land treatment systems for chemical wastes.

7.2.2.5 Photolysis

1. There should be an investigation of photochemical reactions that enhance biodegradation of refractory compounds and those that can produce toxic or undesired breakdown products.

7.2.2.6 Factors Affecting Migration of Contaminants or Their Availability for Biodegradation

1. The mobility of waste organics and their degradation products in soil is not well understood. Evaluation is needed of the extent that petroleum contaminants and their metabolites are sorbed to clay and soil organic matter. Additional information should be developed on the fate and mobility of these wastes in the soil. Information is needed on contaminant sorption and precipitation studies at concentrations in the soil matrix characteristic of those found at remedial action sites. Sorption of contaminants on soil particles can alter their molecular character and enzymatic attack on a given compound. Humic polymers can act as stabilizing agents, making the compounds less resistant to biodegradation. However, the opposite effect has also been observed, with bound material becoming unavailable for biodegradation. More research leading to a better resolution of sorption/biodecomposition interactions is desirable.

2. In addition to descriptions of contaminant sources, prediction of contaminant migration in the saturated zone requires quantitative representations of advection, dispersion, sorption, and transformation that are specific or at least applicable to the site, contaminants, and period of time

in question. Available generalized models for the latter three processes have not yet been convincingly validated using field-scale observations or experiments in even simple sand and gravel aquifers. There is a need for continued research to formulate improved process understanding in models that can be tested at both the laboratory and field scale.

3. The role of spontaneous desorption in governing the rate of bioremediation and possible means of enhancing desorption should be examined.

4. There must be a better understanding of contaminant transport and fate in the vadose zone and in heterogeneous hydrogeologic systems, such as discontinuous and interbedded layers of different geologic media.

5. Evidence from laboratory studies shows that certain organic liquids can cause desiccation and cracking of unweathered clay, which can lead to significant increases in permeability. The significance of this effect in the natural environment is unknown. This should be investigated to see whether it may be an important factor in migration of the contaminants and of any solutions used to treat the site.

6. It is not certain how the rate of dissolution or selective sorption to different soil matrices governs the rate of biodegradation of sparingly soluble organic chemicals in natural ecosystems. Because many synthetic chemicals have low water solubilities, studies should be conducted to assess whether rates of dissolution and desorption govern microbial metabolism in natural conditions.

7. Technologies should be devised to promote bacterial movement through soils or subsurface materials to the site of contamination, and transport of indigenous bacteria with plumes of contamination. Means should be found to overcome the problem of channeling of liquids introduced into the subsurface, whether they contain nutrients, oxygen, or a suspension of bacteria.

7.2.2.7. <u>Effect on Environment</u>

1. More studies are needed to determine the effects of oil on species diversity and the possible influence of chronic or incidental pollution by petroleum hydrocarbons on ecosystem stability. Studies involving the effects of oily wastes on the soil should include studies on soil fauna (e.g., protozoans, worms, insects, and other arthropods), because the overall rate of decomposition can be significantly altered by the reduction in numbers of soil fauna resulting from the addition of toxic materials.

7.2.3 <u>Contaminants</u>

1. Slightly to moderately soluble compounds, such as benzene, will develop a plume in the saturated zone. More information is needed on the interactions among different contaminants in superimposed plumes, the rates and products of biotransformation, the effects of complex contaminant distributions on the activity of microorganisms, and the flow and dissolution of immiscible organic liquids.

2. New long-chain *n*-alkanes (C_{25} to C_{45}) appear during biodegradation of some crude oils as a result of microbial activity and contribute to the formation of tar balls. Reactive biodegradation intermediates may undergo spontaneous head-

to-head condensation reactions, resulting in these very long _n_-alkane chains that are largely immune to further enzymic attack. A clear need exists for further experimental work in this area.

3. The fate of many complex hydrocarbons decomposing in the environment remains to be elucidated. The effects of continued accumulation of partially degraded hydrocarbons on normal microbial functions has not been adequately addressed.

4. Little study has been conducted on degradation of mixtures of many compounds, as would occur in wastes. One compound may prevent or promote the degradation of another in a mixture. The occurrence of multiple waste residues in soil provides the opportunity for the formation of complex or hybrid residues. Our knowledge of such interactions occurring in soil is very limited at present and needs to be expanded.

5. The products of biotransformation of organic pollutants should be identified to be able to assess their toxicity and possible deleterious effects on human health or on populations of animals or plants. Some products may persist in, or immediately adjacent to, the site at which the bioremediation is taking place. Some of the products are toxic and are greater hazards than the original compounds. Characterization should be made of these products and their behavior in the environment to assure minimum or acceptable risks from bioremediation.

6. PAHs containing more than three aromatic rings are very difficult to degrade using existing biodegradation approaches. Further research is needed to develop methods that are appropriate for the removal of recalcitrant PAHs from the environment.

7. Methods should be found to reduce the toxicity of organic or inorganic materials to the microorganisms involved in biodegradation.

8. Use of microorganisms for treatment of mixtures of wastes containing organic and inorganic components should be investigated.

9. Research should be conducted on means to biodegrade chemicals present in nonaqueous-phase liquids. A chemical might become resistant to biodegradation because it is in a nonaqueous phase into which microorganisms or their enzymes may not penetrate. Such compounds appear only slowly in the free water phase and represent long-term pollutants.

10. Special attention should be given to water-insoluble compounds, which may present problems different from those in solution.

11. Research is needed to determine why organic contaminants may not be available for microbial attack. The chemical may be in a form that is not readily utilized.

12. The effects of bioremediation on the partitioning, mobility, and fate of other contaminants present at the same site should be studied.

7.2.3.1 Trace Organics

1. The mechanisms involved and the extent of microbial degradation of trace organics should be further investigated through laboratory and field studies. If the concentration is too low to support growth or may support growth at too slow a rate, methods should be found to bring about a more rapid destruction of the chemical.

7.2.3.2 Metals

1. Despite concern over the environmental impact of heavy metals contained in nearly all major wastes, there are few studies on the effects of these pollutants on microorganisms in their natural environment. Research is needed to evaluate the effects of single and multiple metals (representative of those in petroleum wastes) on microbial processes pertinent to hydrocarbon transformations, such as respiration, nitrogen mineralization, and pertinent enzyme activities. Metal tolerances between different groups of organisms have been observed; however, additional studies are needed to characterize the mechanism by which heavy metals affect metabolism of residues in soils.

2. The nature of trace metal transformations and interactions in microbial degradation processes must be elucidated to prevent or anticipate waste or trace metal overloadings, as could occur during landtreatment, which would inhibit normal soil biodegradation processes.

3. Research is needed to evaluate ways and means of alleviating the toxic effects of metals in soils by addition of organic amendments, such as crop residues, municipal wastes, composts, and by pH adjustments.

4. Information is needed on behavior of specific metals and metal species, i.e., arsenic, beryllium, silver, selenium, mercury, and chromium, in petroleum-contaminated soil systems.

5. More information concerning the factors governing the competition of clays and organic matter for the binding of metals needs to be obtained through research in order to predict the long-term fate of wastes in soils.

6. Research is needed into bioconcentration of metals by microbial cells.

7. Studies should be conducted to possibly exploit the use of oxidation, reduction, and solubilization reactions to promote bioremediation of sites contaminated with toxic metals.

7.2.4 Additives

1. The use of surfactants either alone (to flush otherwise insoluble organics) or in combination with other treatments (to solubilize the waste materials and, thereby, promote biodegradation) is a promising avenue for further research. Both synthetic and natural surfactants warrant further attention.

2. Information is needed on the effects of chemical addition (oxidants, reductants, and polymerizing agents) on the soil properties affecting biotreatment.

3. The use of chelating agents may be a very effective means of managing oily wastes contaminated with toxic metal concentrations. Depending upon the specific chelating agent, stable metal chelates may be highly mobile or may be strongly sorbed to the soil (e.g., Tetran). Considerable research is needed in this area.

7.2.5 Bioreclamation Monitoring

1. Thousands of contamination events are probably remediated naturally before the contamination reaches a point of detection. There is a major need to have methods to determine when natural biorestoration is occurring, the stage the restoration process is in, whether enhancement of the process is possible or desirable, and what will happen if natural processes are allowed to run their course.

2. Further research is needed to relate laboratory results to field situations, where the physical, chemical, and hydrogeological constraints on the microbial community will be different.

3. Research is needed to verify actual destruction efficiencies of the wide range of hazardous constituents found in mixed oily wastes. This will also require some real world testing and demonstration studies. If biological treatment can be developed for RCRA and CERCLA wastes, the commercial opportunities should be significant.

4. Unless all the proper conditions are met for a given compound, biodegradation may not occur, or only at low rates. Better methods for evaluating when these conditions are met and how they affect the rate of degradation are clearly needed. Monitoring can be achieved with indirect evidence from laboratory studies, hydrogeological investigations, and chemical sampling in combination with microbial sampling in the field.

7.2.5.1 Testing Efficiency of Biodegradation

1. Plate counts usually underestimate the total number of microorganisms in a subsurface sample and seldom correlate to required metabolic rate data. A rapid, reliable, and inexpensive index of overall metabolic activity in the subsurface environment is needed.

2. Many of the bacteria in subsurface aquatic environments may be dormant, which could account for some of the difference between the indirect (plate, Most Probably Number (MPN)) and direct (AODC) count methods for the same sample. Further characterization of the metabolic capabilities of groundwater bacteria, or the use of mixtures of labeled substrates to measure total active cells by MPN is needed to verify this.

3. Indirect evidence, such as by nitrogen depletion in the contaminated zone and high bacterial populations in soils, has been used to suggest a certain volume of gasoline has been removed from contaminated soil and groundwater. Indirect methods have been employed since it was not possible to obtain direct evidence of the magnitude of the gasoline conversion to bacterial cells. A more accurate means of making such a determination would be useful to assess the effectiveness of the treatment method, as well as to provide evidence to link the disappearance of the contaminant with biodegradation.

4. Phospholipid concentration is a good estimate of metabolically active biomass; it is feasible to use fatty acids as an indicator of microbial biomass and community structure in soils. Research is needed to determine the exact phospholipid content of subsurface bacteria growing under in situ nutrient conditions.

5. There is a need to determine how accurately math simulation models can predict transformations of petroleum wastes and nutrients in soils once the biocidal and inhibitory effects of toxic constituents are overcome. It may be possible to adopt some existing models, with or without modification for making predictions concerning the fate of oily waste components.

6. Many studies that have indicated soil degradation of petroleum hydrocarbons have merely tested for the disappearance of the total hydrocarbons and not of the many individual components or even major groups of contaminants. There is not yet a conclusive method for determining total petroleum hydrocarbons. Better methods need to be developed pertinent to each fuel type. More information is needed on the extent of detoxification that occurs.

7. Protocols are needed to evaluate the effectiveness of the bioremediation, the organisms, or the products used to enhance bioremediation.

8. Performance criteria should be established to provide standards for evaluating bioremediation technologies. With no standard of achievement against which to optimize the processes, research and development are impeded. Performance criteria would also allow different products or technologies to be compared.

9. Better protocols are needed for quality assurance/quality control of laboratory, microcosm, and field tests.

10. There should be a method for determining the concentration of chemicals remaining after bioremediation.

7.2.6 Process Applications

1. To date, the application of permeable treatment beds at hazardous waste sites has not been performed. However, bench- and pilot-scale testing has provided preliminary quantification of treatment bed effectiveness. There may also be a possibility of inoculating such beds with organisms to create a biofilm over which the contaminated groundwater would pass. Because of potential saturation of bed material, plugging of bed with precipitates, and the short life of treatment materials, this treatment might have to be used as a temporary remedial action rather than a permanent one. These possible limitations should be further investigated. The great variety of infill that could be used in these trenches offers the possibility of specifically targeting a particular contaminant or groundwater condition and could be very useful in pretreatment of a contaminated plume.

2. Indigenous microorganisms that secrete oil repelling polymers could be stimulated to grow in situ to create a biological barrier to contaminant movement.

7.2.7 Microcosms

1. Microcosms are needed to evaluate organisms, treatments, or technologies to promote bioremediation. When appropriately designed to simulate field conditions, microcosms will permit a better evaluation of the effectiveness of the different approaches being studied.

2. Microcosms would be beneficial for studying the fate of pathogens and the fate of chemicals in groundwater. Such microcosms should simulate groundwater conditions.

7.2.8 Bioreactors

1. The kinetics of biodegradation in bioreactors should be studied for pollutants that are destroyed as a direct result of microbial growth on the compounds and for pollutants that serve as secondary substrates. Kinetic expressions should also be developed for compounds at trace levels and at levels that are somewhat inhibitory to the biodegradation process.

2. Use of a bioreactor could be convenient for assessing the value of anaerobic composting to destroy compounds that are resistant to aerobic processes. Optimal conditions for this process should be established.

3. The conditions necessary for destruction of toxic chemicals could be more easily established by using a bioreactor. This controlled environment could investigate the effect of microbial mixtures, levels of nutrients, electron acceptors, aeration, pH, etc. Reactor design and operating conditions should allow for changing biological and reactor conditions. The essential engineering factors should be determined, evaluated, optimized, and demonstrated.

4. Anaerobic bioreactors may allow optimization of conditions for degradation of recalcitrant compounds, increase in degradation rates, and prevention of accumulation of products that are environmentally unacceptable. Criteria should be developed to permit evaluation of whether aerobic or anaerobic processes are more appropriate for particular needs.

5. Bioreactors could be used to study sequential treatments, such as aerobic-anaerobic systems for compounds that are not readily or totally degraded under either condition alone. The sequence could also include preceding biodegradation with a physical, chemical, or photochemical treatment.

7.2.9 Field Applications

1. There is a general lack of information on remedial applications. This includes general information about the technologies in any setting, remedial action, land treatment, agriculture, etc., and specific information on individual technologies. Data are needed on the capacity of soil systems to support in-place technologies, as well as data on ways to enhance the natural capacity by the addition of reagents or modification of the soil/waste system.

2. Information is needed from controlled experiments on a field scale, comparing effectiveness of adding treatment agents (e.g., chemicals, microorganisms, adsorbents) to promote biodegradation, with the effectiveness

of natural processes (e.g., degradation, transformation, immobilization) for soil treatment of constituents in complex hazardous wastes.

3. Although degradation of petroleum hydrocarbons has been extensively studied in the laboratory over the last 25 years, extensive data on full-scale field studies is seldom available or published, and much of the experience rests with experts who have a financial interest in the technology. Publication of the results of the applied methodologies would allow the procedures to be validated and would benefit further development of the technology.

4. A well-controlled test site for research on in situ methods of groundwater decontamination would be very valuable. Such a site could involve containment of groundwater flow (or careful, known constriction). Facilities could be available for conducting physical and chemical process testing, either within an aquifer or at an above-ground collection point. Such a site could either be at an actual contaminated aquifer or at a specifically constructed facility. Establishment of an in situ test site would be advantageous to agencies having parallel interests. It would permit investigations and process development under controlled field conditions and over the period of time required for pilot-scale verification of concept.

5. In open (in situ) systems, the effectiveness of the technology is often influenced by poorly controllable environmental conditions, and the supplemented organisms may not survive. Methods must be developed to facilitate control of the optimum conditions required in each remedial situation.

6. The potential for combinations of chemical and biological treatment methods for accelerating treatment of contaminated soil and groundwater should be explored.

7. Information is needed from the controlled application of complex hazardous wastes (including solid, liquid, and semi-solid wastes) to soil systems in laboratory and field experiments. The data must specifically include information on degradation, mobilization, volatilization, photodegradation, and reaction rates.

8. The problems in moving from the laboratory to field testing should be defined. Factors that constrain field demonstration and evaluations must be overcome. After the feasibility of bioremediation has been established in well-characterized microcosms or similar controlled systems that are sufficiently simple to permit definitive conclusions to be drawn, field demonstrations are needed to establish the problems involved in system design and operation.

9. Appropriate monitoring techniques should be employed to evaluate the effectiveness of the bioremediation.

7.2.10 Site Problems

1. Standard procedures are necessary to ensure that meaningful site samples are collected for analysis to assess the effectiveness of bioremediation at different locations in the field.

2. The conditions and methods appropriate for bioremediation of wastes that are deeply buried should be determined.

3. It is necessary to be able to predict the effect of differences among sites on the effectiveness of bioremediation. A practice that works well at one site may not work well or fail at another. Methods must be established to compare and explain results between different locations.

7.2.11 Case Histories

1. Further examination of case histories is necessary to improve the predictive understanding of the fate of oil pollutants in the environment and the role of microorganisms in biodegradative environmental decontamination.

7.2.12 Funding

1. Neither Federal agencies nor the commercial sector has made a substantial long-term investment in biodegradation research. Few biotechnology companies are entering this field and most companies will not fund biotechnology research on waste treatment. Funding has been directed toward the microorganisms, with little emphasis on developing the process technology. More involvement by Government agencies and industry would help expand and hasten efforts to produce a viable biodegradation technology.

SECTION 7

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